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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/31, C07K 14/205, C12Q 1/68, G01N 33/68, A61K 39/106, C07K 16/12

A1

(11) International Publication Number: WO 98/42842

(43) International Publication Date: 1 October 1998 (01.10.98)

(21) International Application Number: PCT/CA98/00272

(22) International Filing Date: 25 March 1998 (25.03.98)

(30) Priority Data:
60/041,200 25 March 1997 (25.03.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/041,200 (CIP)
Filed on 25 March 1997 (25.03.97)

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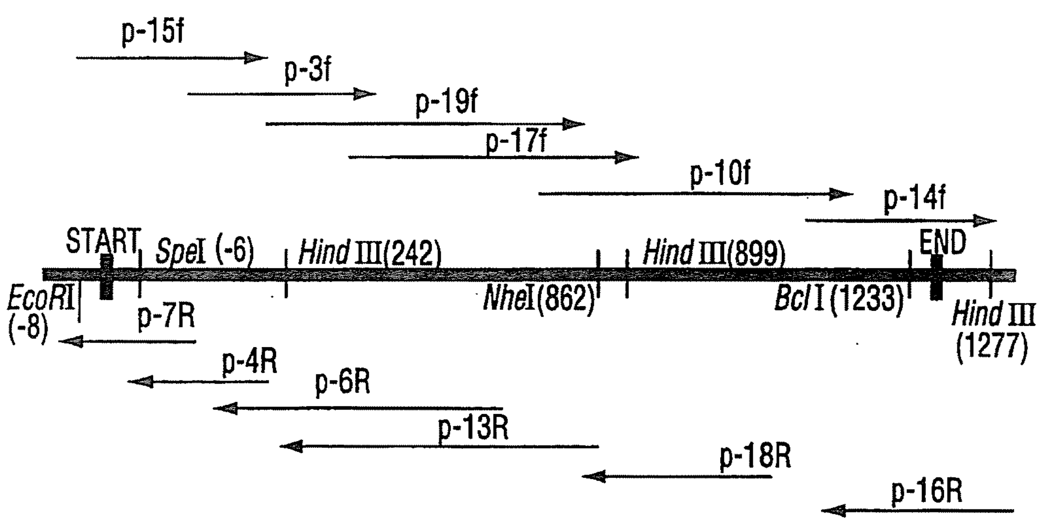
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: A PORIN GENE FROM *CAMPYLOBACTER JEJUNI*, RELATED PRODUCTS AND USES THEREOF



(57) Abstract

The invention relates to a porin gene from *Campylobacter jejuni* [SEQ ID NO:3]. The gene has been designated *porA* and is 1275 bp in length and expresses a protein of 45.6 kDa having a pI of 4.44 [SEQ ID NO:2]. The sequencing and cloning of the gene makes possible various medical and industrial uses. For examples, knowledge of the DNA code makes it possible to design DNA probes for identification of the gene in samples for testing. A positive result indicates the presence of the gene in the sample and is a strong indicator of the presence of *C. jejuni*. Such probes can also be used to isolate the corresponding cDNA, that may then be amplified by polymerase chain reaction. The development of DNA probes based on a known sequence is a known procedure that is familiar to persons skilled in the art and it will be possible for such persons to develop suitable probes without undue experimentation. Normally, such probes would consist of at least 15 consecutive nucleotides from the cDNA sequence.

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A PORIN GENE FROM *Campylobacter jejuni*, RELATED PRODUCTS AND USES THEREOF

TECHNICAL FIELD

This invention relates to a porin gene from *Campylobacter jejuni*, to related products and to the uses

BACKGROUND ART

In the following discussion, the numbers shown in brackets refer to the articles identified in the "REFERENCES" section provided later in this specification.

Campylobacter jejuni is recognized as a cause of bacterial-induced diarrhea in both developing and underdeveloped countries (39, 41). Active surveys conducted in the United States have estimated the number of cases of campylobacteriosis to be 2.5 million per year, making it a multi-million dollar disease (39). Symptoms caused by *C. jejuni* can range from watery to bloody diarrhea (28, 39). In most cases campylobacteriosis is a self-limiting disease but in the more severe cases, antibiotic intervention with macrolids or fluoroquinolones or rehydration therepy is necessary to eradicate the infection (28).

The organism has been reported to possess several virulence factors that may be responsible for disease (11, 26, 40) but little is known regarding the genetic processes that surround their production. One virulence factor, a toxin, has been cloned and sequenced successfully and this is the cytolethal distending toxin (CLDT) of *C. jejuni* (34). The CLDT operon was found to contain three open reading frames (ORF) designated *cdtA*, *cdtB* and *cdtC* and these correspond with 30.1 kDa, 28.9 kDa and 21.1 kDa proteins respectively (34). *Escherichia coli* minicell experiments have shown that all three genes are necessary for the production of the active toxin. Screening of multiple strains of *Campylobacter* sp. by

polymerase chain reaction (PCR) for the presence of the *cdtB* gene and HeLa cell assays for the expression of CLDT revealed that all the strains tested carried the gene and tested positive in the cell culture assay (34). Johnson and Lior (19) originally reported that 41% of 718 isolates of *Campylobacter* sp. screened for the production of CLDT were positive; however, isolates screened for the *cdtB* gene suggested that this percentage may be higher than was previously reported (19, 34). Genetic studies involving the production of an enterotoxin by *C. jejuni* revealed DNA similarities between a postulated GM_1 binding site on the *toxB* gene from *Vibrio cholerae* and the *eltB* gene from *E. coli*. Despit this an enterotoxin gene has not been successfully cloned and sequenced from *C. jejuni* (5).

C. jejuni has a genome estimated to be 1.7 Mb in size as determined by pulsed field gel electrophoresis (PFGE) (43) while the a percentage of guanidine+cytosine ranged between 29-36 mol % (42, 43). The organism has the capability to transform free DNA as well as to be transduced by bacteriophages and to transfer DNA between strains by conjugation (42, 44). These genetic exchange mechanisms could facilitate the spread of antibiotic resistant determinants between strains (44) and may result in the aquistion of toxin production by one strain from anther (32). A number of genes have been sequenced from *C. jejuni* (42); however, the majority of these take the form of highly conserved or "housekeeping" genes such as serine hydroxylmethyl-transferase (*glyA*) (7) and (-glutamyl phosphate reductase gene (*proA*) (22). In addition, genes such as *flaA* and *flaB* encoding flagella proteins (15) and *peb4A*, an antigenic surface protein (4) have been cloned and sequenced. Difficulties such as gene instability and failure to express functional products

have been encountered and this has made genetic analysis of *C. jejuni* problematic (34, 42).

Several porin genes from various bacterial species have been purified, cloned and sequenced (6, 14, 16,17, 27). These porin usually exist as a single monomeric protein (16, 29) or homotrimers (3, 6) and all show a variation in their relative pores sizes (3, 17). Porins are functional components of the outer membrane of bacteria and they allow for the exchange of solutes as well as permit the excretion of waste products to occur. One characterized porin from *E. coli* has been found to occur at a frequency of 10^5 on each bacterial cell (27, 46) making it the most abundant molecule present on the cell surface (36, 46). Porins have also been found to induce morphologic changes in HEp-2 cells as a result of alterations in the cytoskeleton following incubation with increasing concentration of the purified protein (8).

The major outer membrane protein (MOMP) of *C. jejuni* was first isolated and reconstituted into lipid bilayer membranes and found to form small channels consistent with that of a porin (18). The MOMP has an apparent molecular weight of 45 kDa under native conditions and since 3-folder monomers are needed to form the functional porin it was confirmed to be part of the trimeric porin family (3). The N-terminal sequence has been elucidated and been found to contain little homology with other bacterial porin proteins (3) but it did share homology with two outer membrane proteins from *W. recta* (20). In this thesis it was reported that the porin-LPS complex from *C. jejuni* possessed a heat-labile cytotoxic activity and was capable of inducing apoptosis in HEp-2 cells but not in Vero cells.

Thus, while the characterization of the protein with respect to its pore capabilities has already been reported (18), the corresponding gene and its sequence have not previously identified.

DISCLOSURE OF THE INVENTION

An object of the present invention is to identify and sequence the a porin gene of *C. jejuni* responsible for the production of a cytotoxic protein-LPS complex so that the gene can be cloned and expressed, and so that useful products and methods can be developed.

Another object of the invention is to identify a gene responsible for cytotoxic activity of *C. jejuni* to facilitate the identification and treatment of infections of mammals by the organism, and to enable prophylaxis against such infection.

According to one aspect of the invention, there is provided an isolated and purified *porA* gene from *Campylobacter jejuni*, characterized in that said gene expresses a 424 amino acid cytotoxic protein having a calculated molecular weight of 45.6 kDa and a pI of 4.44.

Another aspect of the invention comprises a DNA probe, characterized in that said probe has a nucleotide sequence corresponding to a part of a target sequence SEQ ID NO:1, wherein the nucleotide sequence of the probe encompasses nucleotide substitutions, additions and deletions that do not affect the ability of the probe to bind specifically to said target.

The invention also relates to a method of detecting the presence of *Campylobacter jejuni* infection, characterized by the steps of: a) contacting a sample obtained from a patient suspected of infection, with a detectable amount of a purified cytotoxic rotein encoded by at least a portion of the nucleic acid of the invention, for a time sufficient to allow formation of a complex between said protein and any anti-*Campylobacter jejuni* antibodies present in said sample; and b) detecting the presence of, and optionally the quantity of, said complex formed during step (a).

In another form, the invention comprises an isolated expression vector, characterized by a region encoding a

porA protein of Campylobacter jejuni, or an antigenic fragment thereof.

Included within the invention is a method of inducing an immune response in a human or animal host by administering to the host a foreign protein, characterized in that said protein has an amino acid sequence SEQ ID NO:2, wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the ability of the protein to raise antibodies when introduced into said human or animal body.

Yet another aspect of the invention is a method of producing antibodies for testing for infection by *Campylobacter jejuni*, characterized in that a protein having an amino acid sequence of SEQ ID NO:2 is introduced into a human or animal body to raise antibodies, and said antibodies are subsequently isolated from said body, wherein said amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the ability of the protein to raise antibodies when introduced into said human or animal body.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the sequencing reactions and restriction map of *porA* from C. jejuni strain 2483 showing the restriction sites for the enzymes used in the generation of a vectorette library (the arrows designate the direction and primer used in the sequencing of the intact gene).

Figure 2 shows a southern blot analysis of genomic digests using a digoxigenin-labeled 650 bp probe. Lanes 1 and 10: *Hind* III digested lambda DNA; Lane 2: *Hind* III digested C. *jejuni* genomic DNA; Lane 3: *Bam*HI digested *C. jejuni* genomic DNA; Lane 4: *Bgl* I digested *C. jejuni* genomic DNA; Lane 5: *Nhe* I digested *C. jejuni* genomic DNA; Lane 6: *Eco*RI digested *C. jejuni* genomic DNA; Lane 7: *Bcl* I digested *C. jejuni* genomic DNA; Lane 8: *Spe* I digested

C. jejuni genomic DNA; Lane 9: *E. coli Xba* I digested genomic DNA.

Figure 3 shows the complete open reading frame and translated protein of the porA gene. The single underlined sequence represents the putative Shine-Dalgarno ribosome binding site (RBS), -10 and -35 sequences, and double lines represents a stem loop structure which may indicate a rho-independent transcription termination site with a 5 bp loop followed by a poly-T region of DNA. Bold face letters represent the initiation codon and "*" represents the termination codon and the numbering is for the nucleotide and amino acid count.

Figure 4 shows alignment of *C. jejuni* PorA with *H. influenzae* P2, *E. cloacae* PhoE, *K. pneumoniae* PhoE, *S. typhi* OmpC, and *E. coli* PhoE using GCG (Genetics Computer Group). Capital letters represent identical or conserved changes, small letters represent mismatches in the sequences and spaces (...) were inserted in order to achieve the best alignment. "*" represents termination codon.

Figure 5 shows a stem loop structure of the termination sequence of the *porA* gene. The numbering represents the position of the loop in the 1450 bp fragment of Fig. 3.

Figure 6A shows morphological changes induced in HEp-2 cells after 48 h treatment with *C. jejuni* cytotoxic porin-LPS complex for control cells;

Figure 6B shows the morphological changes for cells intoxicated with 1 μg of isolated *C. jejuni* cytotoxic complex; note cytoplasmic vacuoles (arrowed);

Figure 6C shows the morphological changes for cells intoxicated with 10 μg of isolated *C. jejuni* cytotoxic porin-LPS complex. (magnification X 150);

Figure 7A shows an elution profile and silver stain of the cytotoxic complex for a fraction from a G75 gel filtration column;

Figure 7B shows an elution profile and silver stain of the cytotoxic complex for a fractionation of peak A on a TSK DEAE-5PW column. +++, >70% of Hep-2 cells rounded by 48 h; ++50-70% cell rounded by 48 h; +, <50% cell rounded by 48 h;

Figure 8 shows western blot analysis of the isolated cytotoxic porin-LPS complex from *Campylobacter sp* using 40 μg of crude, concentrated filtrate and homologous rabbit antiserum. Lanes 1 and 9: Prestained standards (kDa) (Gibco BRL); Lane 2: uninoculated broth; Lane 3: *Aeromonas veronii* LCDC A2297 (used as a negative control); Lane 4: *C. coli* strain 8682; Lane 5: *C. jejuni* LCDC 16336; Lane 6: *C. jejuni* LCDC 3969; Lane 7: *C. jejuni* strain 2483; Lane 8: *E. coli* (VT1) LCDC 3787.

Figure 9 shows double staining of native-PAGE (lanes 1 and 2) and SDS-PAGE (lanes 3 and 4) gels with periodic acid Schiff (PAS) and Coomassie blue. Lane 1: native low molecular weight standards (Pharmacia); Lane 2: 10 μg of native carbohydrate co-purified with *C. jejuni* isolated cytotoxic porin-LPS complex; Lane 3: 10 μg of heat denatured carbohydrate which co-purified with *C. jejuni* isolated cytotoxic porin-LPS complex; Lane 4: kaleidoscope prestained standards (kDa) (BioRad).

Figure 10 shows western blot analysis of the isolated cytotoxic complex with the lectin GNA. Lanes 1 and 4: kaleidoscope prestained standards (kDa) (BioRad); Lane 2: 10 μg of carbohydrate from the isolated *C. jejuni* cytotoxic porin-LPS complex; Lane 3: 15 μg carboxypeptidase Y;

Figure 11A shows hydrophobic profiles and beta sheet propensities as determined by the method of Novotny using PC/Gene software package for *C. jejuni* strain 2483 PorA;

Figure 11B shows the hydrophobic profiles and beta sheet propensities for *H. influenzae* P2; and

Figure 11C shows the hydrophobic profiles and beta sheet propensities for *C. jejuni* FlaA.

BEST MODES FOR CARRYING OUT THE INVENTION

The present invention is based on the identification of a porin-lipopolysaccharide (LPS) complex from *Campylobacter jejuni* that is an endotoxin and that is fairly well conserved amongst strains of the organism, but not widely found in other *Campylobacter* species. The complex has been isolated and a corresponding porin gene, designated "*porA*," has been identified, isolated, sequenced and cloned by the inventors of the present invention.

Specifically, the complex was obtained from strain 2483 of *C. jejuni*. While this strain is common in nature and can be identified by designing a suitable probe from the sequence disclosed herein, the inventors and assignee of this application have deposited a sample of the strain with the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852 USA. The deposit was made on March 19, 1998 under the terms of the Budapest Treaty, and has been awarded the accession no. ATCC 202,101.

The sequencing and cloning of the gene makes possible various medical and industrial uses. For example, knowledge of the DNA code makes it possible to design DNA probes for identification of the gene in samples for testing. A positive result indicates the presence of the gene in the sample and is a strong indicator of the presence of *C. jejuni*. Such probes can also be used to isolate the corresponding cDNA, that may then be amplified by polymerase chain reaction. The development of DNA probes based on a known sequence is a known procedure that is familiar to persons skilled in the art and it will be possible for such persons to develop suitable probes without undue experimentation. Normally, such probes

would consist of at least 15 consecutive nucleotides from the cDNA sequence.

Furthermore, expression of the gene, or a significant part thereof, in a suitable transformed host (e.g. transformed *E. coli* or the like) makes it possible to produce usable quantities of an expressed protein that induces the immune system to raise antibodies. This can be used to vaccinate the host against the effects of intoxication with *C. jejuni* without causing harmful effects. For this purpose, the protein may be used in conjunction with suitable pharmacuetically-acceptable carriers and may be used in concentrations of the protein in the composition to achieved the desired protective effect. Suitable modes of administration may be employed, e.g. oral or parenteral administration.

The protein can also be used to produce antibodies (e.g. in rabbit) useful in testing blood samples for patients infected with *C. jejuni*.

It will be appreciated by persons skilled in the art that the sequence of the *porA* gene identified herein may undergo modification by substitution, addition or deletion of a certain number of nucleotides without affecting the uses of the present invention indicated above. The present invention therefore also extends to isolated and purified nucleic acid exhibiting such substitutions, additions or deletions, and expression products thereof, and probes designed for the identification thereof.

The teachings of International (PCT) Patent Publication No. WO 95/05850 (published on March 2, 1995; inventor: Martin J. Blaser; Applicant: Enteric Research Laboratories, Inc) are also relevant to the isolation and uses of the *porA* gene and the products derived therefrom. The following information and procedures are specifically mentioned.

The "isolated" nucleic acid is separated from other nucleic acids found in the naturally occurring organism.

This specific nucleic acid can be used to detect C. jejuni possessing the *porA* antigen in methods such as polymerase chain reaction, ligase chain reaction and hybridization.

The isolated sequence or appropriate fragments thereof can be utilized to produce a *porA* protein, by splicing the sequence into an appropriate vector and transfecting an appropriate host. In addition, the nucleic acid can be homologous with nucleotide sequences present in other bacteria. Such an amino acid sequence shared with other bacteria can be used for example to simultaneously detect related strains or as a basis for a multiprotective vaccine.

An isolated nucleic acid capable of selectively hybridizing with or selectively amplifying a nucleic acid encoding the *porA* antigen or fragments thereof is also contemplated. An isolated nucleic acid complementary to the above nucleic acid is also provided. The sequences can be selected based on the nucleotide sequence and the utility of the particular sequence.

Modifications to the nucleic acids of the invention are also contemplated as long as the essential structure and function of the polypeptide encoded by the nucleic acids is maintained. Likewise, fragments used as primers or probes can have substitutions so long as enough complementary bases exist for selective hybridization.

Purified antigenic polypeptide fragments encoded by the nucleic acids of the present invention are also contemplated. The "purified" antigen is sufficiently free of contaminants or cell components with which the antigen normally occurs to distinguish the antigen from the contaminants or components.

An antigenic fragment of the antigen can be isolated from the whole antigen by chemical or mechanical disruption. The purified fragments thus obtained can be tested to determine their antigenicity and specificity by the methods taught herein. Antigenic fragments of the antigen can also be synthesized directly. An

immunoreactive fragment is an amino acid sequence of at least abut 5 consecutive amino acids derived from the *PorA* antigen.

The polypeptide fragments of the present invention can also be recombinant proteins obtained by cloning nucleic acids encoding the polypeptide in an expression system capable of producing the antigenic polypeptide or fragments thereof.

Once the amino acid sequence of the antigen is provided, it is also possible to synthesize, using standard peptide synthesis techniques, peptide fragments chosen to be homologous to immunoreactive regions of the antigen and to modify these fragments by inclusion, deletion or modification of particular amino acids residues in the derived sequences. Thus, synthesis or purification of an extremely large number of peptides derived from the antigen is possible.

The amino acid sequences of the present polypeptides can contain an immunoreactive portion of *PorA* antigen attached to sequences designed to provide for some additional property, such as solubility. The amino acid sequences of an *PorA* antigen can include sequences in which one or more amino acids have been substituted with another amino acid to provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase its biolongevity, alter enzymatic activity, or alter interactions with gastric acidity. In any case, the peptide must posses a bioactive property, such as immunoreactivity, immunogenicity, etc.

Determining Immunogenicity

The purified polypeptide fragments thus obtained can be tested to determine their immunogenicity and specificity by techniques known in the art. Various concentrations of a putative immunogenically specific fragment are prepared and administered to an animal and the immunological response (e.g., the production of antibodies or cell mediated immunity) of an animal to each concentration is determined. The amounts of antigen administered depend on the subject e.g. a human or a guinea pig, the condition of the subject, the size of the subject, etc. Thereafter an animal so inoculated with the antigen can be exposed to the bacterium to test the potential vaccine effect of the specific immunogenic fragment. The specificity of a putative immunogenic fragment can be ascertained by testing sera, other fluids or lymphocytes from the inoculated animal for cross reactivity with other closely related bacteria.

Vectors and Hosts

A vector comprising the nucleic acids of the present invention is also provided. The vectors of the invention can be in a host capable of expressing the antigen. There are numerous *E. coli* expression vectors known to one of ordinary skill in the art useful for the expression of the antigen. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilus*, and other *enterobacteriaceae*, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species.

In these prokaryotic hosts one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a

promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences for example, for initiating and completing transcription and translation. If necessary an amino terminal methionine can be provided by insertion of a Met codon 5' and in-frame with the antigen. Also, the carboxyl terminal extension of the antigen can be removed using standard oligonucleotide mutagenesis procedures.

Additionally, yeast expression can be used. There are several advantages to yeast expression systems. First, evidence exists that proteins produced in a yeast secretion systems exhibit correct disulfide pairing. Second, post-translational glycosylation is efficiently carried out by yeast secretory systems. The Saccharomyces cerevisiae pre-pro-alpha-factor leader region (encoded by the MFα-1 gene) is routinely used to direct protein secretion from yeast (Brake et al., 1984). The leader region of pre-pro-alpha-factor contains a signal peptide and a pro-segment which includes a recognition sequence for a yeast protease encoded by the KEX2 gene: this enzyme cleaves the precursor protein on the carboxyl side of a Lys-Arg dipeptide cleavage-signal sequence. The antigen coding sequence can be fused inframe to the pre-pro-alpha-factor leader region. This construct is then put under the control of a strong transcription promoter, such as the alcohol dehydrogenase I promoter or a glycolytic promoter. The antigen coding sequence is followed by a translation termination codon

which is followed by transcription termination signals.

Alternatively, the antigen coding sequences can be fused to a second protein coding sequence, such as Sj26 or β galactosidase, used to facilitate purification of the fusion protein by affinity chromatography. The insertion of protease cleavage sites to separate the components of the fusion protein is applicable to constructs used for expression in yeast.

Mammalian cells permit the expression of proteins in an environment that favors important post-translational modifications such as folding and cysteine pairing, addition of complex carbohydrate structures, and secretion of active protein. Vectors useful for the expression of antigen in mammalian cells are characterized by insertion of the antigen coding sequence between a strong viral promoter and a polyadenylation signal. The vectors can contain genes conferring either gentamicin or methotrexate resistance for use as selectable markers. The antigen and immunoreactive fragment coding sequence can be introduced into a Chinese hamster ovary cell line using a methotrexate resistance-encoding vector. Presence of the vector DNA in transformed cells can be confirmed by Southern analysis and production of an RNA corresponding to the antigen coding sequence can be confirmed by Northern analysis. A number of other suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoflogulin genes, SV40, Adenovirus, and Bovine Papilloma Virus, etc. The vectors containing the DNA segments of interest can be transferred

into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego, CA ("MaxBac"™ kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987) (hereinafter "Summers and Smith"). Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, inter alia, Aedes aegypti, Autographa Californica, Bombyx mori, Drosophila melanogaster, Spodoptera frugiperda, and Trichoplusia ni (PCT Pub. No. WO 89/046699; Carbonell et al., J. Virol., 56:153 (1985); Wright, Nature, 321:718 (1986); Smith et al., Mol. Cell. Biol., 3:2156 (1983), and see generally, Fraser, et al., In vitro Cell. Dev. Biol., 25:225 (1989).

Alternative vectors for the expression of antigen in mammalian cells can also be employed, e.g those similar to those developed for the expression of human gamma-interferon, tissue plasminogen activator, clotting Factor VIII, hepatitis B virus surface antigen, protease Nexinl, and eosinophil major basic protein. Further, the vector can include CMV promoter sequences and a polydenylation signal available for expression of inserted DNAs in mammalian cells (such as COS7).

The DNA sequences can be expressed in hosts after the sequences have been operably linked to, i.e., positioned to ensure the functioning of, an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, a

selectable marker such as genes for tetracycline resistance or hygromycin resistance are utilized to permit detection and/or selection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

Polynucleotides encoding a variant polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

Purified Antibodies

A purified monoclonal antibody specifically reactive with *PorA* is also provided. The antibodies can be specifically reactive with a unique epitope of *PorA* or they can also react with epitopes of other organisms. The term "reactive" means capable of binding or otherwise associating nonrandomly with an antigen. "Specifically reactive" as used herein describes an antibody or other ligand that does not cross react substantially with any antigen other than the one specified, in this case, usually *PorA* antigen, or antigenic fragments thereof. Antibodies can be made as described in the Examples (see also, Marlow and Lane, Antlbodies; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988). Briefly, purified antigen can be injected into an animal in an amount and in intervals sufficient to elicit an immune response. Antibodies can either be purified directly, or spleen cells can be obtained from the animal. The cells are then fused with an immortal cell line and screened for antibody secretion.

The antibody can be bound to a substrate or labeled with a detectable moiety, or both bound and labeled. The detectable moieties contemplated with the composition of the present invention are those listed below in the description of the diagnostic methods, including fluorescent, enzymatic and radioactive markers.

Antigen Bound to Substrate

A purified *PorA* antigen bound to a substrate and a ligand specifically reactive with the antigen are also contemplated. Such a purified ligand specifically reactive with the antigen can be an antibody. The antibody can be a monoclonal antibody obtained by standard methods and as described herein. The monoclonal antibody can be secreted by a hybridoma cell line specifically produced for that purpose (Harrow and Lane, 1988). Likewise, nonhuman polyclonal antibodies specifically reactive with the antigen are within the scope of the present invention. The polyclonal antibody can also be obtained by the standard immunization and purification protocols (Harrow and Lane, 1988).

Serological Detection (Diaynosis) Methods Detecting Antibody with the Antigen

The present invention provides a method of detecting the presence of *C. jejuni* strain possessing the *PorA* antigen in a subject, comprising the steps of contacting an antibody-containing sample from the subject with a detectable amount of the *PorA* antigenic fragment of the present invention and detecting the reaction of the fragment and the antibody, the reaction indicating the presence of the *C. jejuni* strain or previous infection with the *C. jejuni* strain.

Detecting Antigen with Antibody/Ligand

One example of the method of detecting *C. jejuni* possessing the *PorA* antigen is performed by contacting a

fluid or tissue sample from the subject with an amount of a purified antibody specifically reactive with the antigen, and detecting the reaction of the ligand with the antigen. It is contemplated that the antigen will be on intact cells containing the antigen, or will be fragments of the antigen. As contemplated herein, the antibody includes any ligand which binds the antigen, for example, an intact antibody, a fragment of an antibody or another reagent that has reactivity with the antigen. The fluid sample of this method can comprise any body fluid which would contain the antigen or a cell containing the antigen, such as blood, plasma, serum, saliva and urine. Other possible examples of body fluids include sputum, mucus, gastric juice and the like.

ELISA

Immunofluorescence assays (IFA) and enzyme immunoassays such as enzyme linked immunosorbent assays (ELISA) and immunoblotting can be readily adapted to accomplish the detection of the antigen. An ELISA method effective for the detection of the antigen can, for example, be as follows: (1) bind the antibody to a substrate; (2) contact the bound antibody with a fluid or tissue sample containing the antigen; (3) contact the above with a secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; (6) observe color change. The above method can be readily modified to detect antibody as well as antigen.

Competitive Inhibition Assay

Another immunologic technique that can be useful in the detection of *C. jejuni* expression *PorA* or previous *C. jejuni* infection utilizes monoclonal antibodies (MAbs) for detection of antibodies specifically reactive with *PorA*

antigen. Briefly, sera or other body fluids from the subject is reacted with the antigen bound to a substrate (e.g. an ELISA 96-well plate). Excess sera is thoroughly washed away. A labeled (enzyme-linked, fluorescent, radioactive, etc.) monoclonal antibody is then reacted with the previously reacted antigen-serum antibody complex. The amount of inhibition of monoclonal antibody binding is measured relative to a control (no patient serum antibody). The degree of monoclonal antibody inhibition is a very specific test for a particular variety or strain since it is based on monoclonal antibody binding specificity. MAbs can also be used for detection directly in cells by IFA.

Micro-Agglutination Assay

A micro-aggulatination test can also be used to detect the presence of the *C. jejuni* strain in a subject. Briefly, latex beads (or red blood cells) are coated with the *PorA* and mixed with a sample from the subject, such that antibodies in the tissue or body fluids that are specifically reactive with the antigen crosslink with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible with the naked eye or by spectrophotometer. In modification of the above test, antibodies specifically reactive with the antigen can be bound to the beads and antigen in the tissue or body fluid thereby detected.

Sandwich Assay/Flow Cytometry/Immunoprecipitation

In addition, as in a typical sandwich assay, the antibody can be bound to a substrate and reacted with the antigen. Thereafter, a secondary labeled antibody is bound to epitopes not recognized by the first antibody and the secondary antibody is detected. Since the present invention provides *PorA* antigen for the detection of *C. jejuni* or previous *C. jejuni* infection, other serological

methods such as flow cytometry and immunoprecipitation can also be used as detection methods.

In the diagnostic methods taught herein, the antigen can be bound to a substrate and contacted by a fluid sample such as serum, urine, saliva or gastric juice. This sample can be taken directly from the patient or in a partially purified form. In this manner, antibodies specific for the antigen (the primary antibody) will be specifically react with the bound antigen. Thereafter, a secondary antibody bound to, or labeled with, a detectable moiety can be added to enhance the detection of the primary antibody. Generally, the secondary antibody or other liyand which is reactive, either specifically with a different epitope of the antigen or nonspecifically with the ligand or reacted antibody, will be selected for its ability to react with multiple sites on the primary antibody. Thus, for example, several molecules of the secondary antibody can react with each primary antibody, making the primary antibody more detectable.

Detectable Moieties

The detectable moiety will allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry, radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change). The detection methods and moieties used can be selected, for example, from a list above or other suitable examples by the standard criteria applied to such selections (Harrow and Lane, 1988).

Treatment Methods

Methods of treating *C. jejuni* enteritis in a subject using the compositions of the present invention are provided. For example, in one such method an amount of ligand specifically reactive with the *PorA* antigen of *C. jejuni* sufficient to bind the antigen in the subject and improve the subject's clinical condition is administered to the subject. Such improvement results from the ligand interfering with the antigen's normal function in inducing cell adherence inflammation and cellular damage. The ligand can be purified monoclonal antibody specifically reactive with the antigen, a purified polyclonal antibody derived from a nonhuman animal, or other reagent having specific reactivity with the antigen. Additionally, cytotoxic moieties can be conjugated to the ligand/antibody by standard methods. Examples of cytotoxic moieties include ricin A chain, diphtheria toxin and radioactive isotopes.

Another method of treating *C. jejuni* enteritis subject comprises administering to the subject an amount of a ligand/antagonist for a receptor for the *PorA* antigen of *C. jejuni* sufficient to react with the receptor and prevent the binding of the *PorA* antigen to the receptor. The result is an improvement in the subject's clinical condition. Alternatively, the treatment method can include administering to the subject an amount of an analogue of a *PorA* receptor to result in competitive binding of the *PorA* antigen, thus inhibiting binding of the *PorA* antigen to its wild type receptor. The receptor is localized on cells present in the intestinal mucosa, such as epithelial cells, inflammatory cells, or endothelial cells.

Vaccines

The *PorA* antigen of this invention can be used in the construction of a vaccine comprising an immunogenic amount

of the antigen and a pharmaceutically acceptable carrier. The vaccine can be the entire antigen, the antigen on an intact *C. jejuni*, E. cold or other strain. The vaccine can then be used in a method of preventing *C. jejuni* infection. As mentioned, supra, mutant forms of *C. jejuni* may also be used.

Immunogenic amounts of the antigen can be determined using standard procedures. Briefly, various concentrations of a putative specific immunoreactive epitope are prepared, administered to an animal and the immunological response (e.g., the production of antibodies) of an animal to each concentration is determined.

The pharmaceutically acceptable carrier in the vaccine of the instant invention can comprise saline or other suitable carriers (Arnon, R. (Ed.) Synthetic Vaccines I:L 83-92, CRC Press, Inc., Boca Raton, Florida, 1987). An adjuvant can also be a part of the carrier of the vaccine, in which case it can be selected by standard criteria based on the antigen used, the mode of administration and the subject (Arnon R. (Ed.), 1987). Methods of administration can be by oral or sublingual means, or by injection, depending on the particular vaccine used and the subject to whom it is administered. It can be appreciated from the above that the vaccine can be used as a prophylactic (to prevent infection) or a therapeutic (to treat disease after infection) modality.

Thus, the invention provides methods of preventing or treating *C. jejuni* infection and the associated diseases by administering the vaccine to a subject.

Such vaccines comprise antigen or antigens, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids,

polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, H. pylori, etc. pathogens.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-inwater emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides, or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween™ 80, and 0.5% Span™ 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer Microfluidics, Newton, MA), t b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL ~ CWS (Detox); (3) saponin adjuvants, such as Stimulon (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant and Incomplete Freunds Adjuvant (IFA); (5) Cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor

necrosis factor (TNF), etc ; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'2'-dipalmitoyl-sn-glycero-3-huydroxyphosphoryloxy)ethylamine (MTP-PE), etc.

The immunogenic compositions (e.g., the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect.

Typical immunogenic compositions used as vaccines comprise-an immunologically effective amount of antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount," it is meant that the administration of that amount to an individual, either in a single does or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical

situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

Nucleic Acid Detection (Diagnosis) Methods

The presence of the *PorA* antigen and *C. jejuni* possessing the *PorA* antigen can also be determined by detecting the presence of a nucleic acid specific for the antigen. The specificity of these sequences for the antigen can be determined by conducting a computerized comparison with known sequences, catalogued in GenBank, a computerized database, using the computer programs Word Search or FASTA of the Genetics Computer Group (Madison, WI), which search the catalogued nucleotide sequences for similarities to the gene in question.

The nucleic acid specific for the antigen can be detected utilizing a nucleic acid amplification technique, such as polymerase chain reaction or ligase chain reaction. Alternatively, the nucleic acid is detected utilizing the direct hybridization or by utilizing a restriction fragment length polymorphism. For example, the present invention provides a method of detecting the presence of *C. jejuni*, possessing the *PorA* antigen, comprising ascertaining the presence of a nucleotide sequence associated with a restriction endonuclease cleavage site. In addition, PCR primers which hybridize only with nucleic acids specific for the antigen can be utilized. The presence of amplification indicates the

presence of the antigen. In another embodiment, a restriction fragment of a DNA sample can be sequenced directly using for example, Sanger ddNTp sequencing or 7-deaza-2'-deoxyguanosine 5'-triphosphate and Taq polymerase, and compared to the known unique sequence to detect *C. jejuni* . In a further embodiment, the present invention provides a method of detecting the presence of *C. jejuni* by selective amplification by the methods described above. In yet another embodiment, *C. jejuni* can be detected by directly hybridizing the unique sequence with a *PorA* selective nucleic acid probe.

Furthermore, the nucleotide sequence could be amplified prior to hybridization by the methods described above.

Once specific sequences are shown to be associated with *C. jejuni*, the methods to detect specific sequences are standard in the art. Detection of specific sequences using direct probing involves the use of oligonucleotide probes which may be prepared, for example, synthetically or by nick translation. The probes may be suitably labeled using, for example, a radio label, enzyme label, fluorescent label, biotin-avidin label and the like for subsequent visualization in the example of Southern blot hybridization procedure. The labeled probe is reacted with a bound sample DNA, e.g., to a nitrocellulose sheet under conditions such that only fully complementary sequences hybridize. The areas that carry DNA sequences complementary to the labeled DNA probe become labeled themselves as a consequence of the reannealing reaction.

The areas of the filter that exhibit such labeling may then be visualized, for example, by autoradiography. The label probe is reacted with a DNA sample bound to, for example, nitrocellulose under conditions such that only fully complementary sequences will hybridize. The stringency of hybridization is usually ioc below the Ti (the irreversible melting temperature of the hybrid formed

between the probe and its target sequence) for the given chain length. For 20mers, the recommended hybridization temperature is about 58°C. The washing temperatures are unique to the sequence under investigation and need to be optimized for each variant.

Alternative probing techniques, such as a ligase chain reaction (LCR), involve the use of mismatch probes, i.e., probes which are fully complementary with the target except at the point of the mutation. The target sequence is then allowed to hybridize both with oligonucleotides which are fully complementary and have oligonucleotides containing a mismatch, under conditions which will distinguish between the two. By manipulating the reaction conditions, it is possible to obtain hybridization only where there is full complementarily. If a mismatch is present, there is significantly reduced hybridization.

The polymerase chain reaction (PCR) is a technique that amplifies specific DNA sequences with remarkable efficiency. Repeated cycles of denaturation, primer annealing and extension carried out with polymerase, e.g., a heat stable enzyme Taq polymerase, leads to exponential increases in the concentration of desired DNA sequences. Given a knowledge of the nucleotide sequence of a mutation, synthetic oligonucleotides can be prepared which are complementary to sequences which flank the DNA of interest. Each oligonucleotide is complementary to one of the two strands. The DNA can be denatured at high temperatures (e.g., 95°C) and then reannealed in the presence of a large molar excess of oligonucleotides. The oligonucleotides, oriented with their 3' ends pointing towards each other, hybridize to opposite strands of the target sequence and prime enzymatic extension along the nucleic acid template in the presence of the four deoxyribonucleotide triphosphates. The end product is then denatured again for another cycle. After this three-step cycle has been repeated several times, amplification of a

DNA segment by more than one millionfold can be achieved. The resulting DNA may then be directly sequenced in order to locate any genetic alteration. Alternatively, it may be possible to prepare oligonucleotides that will only bind to altered DNA, so that PCR will only result in multiplication of the DNA if a mutation is present. Following PCR, direct visualization of allele-specific oligonucleotide hybridization may be used for typing *C. jejuni* strain associated with an outbreak.

Alternatively, an adaptation of PCR called amplification of specific alleles (PASA) can be employed; this uses differential amplification for rapid and reliable distinction between alleles that differ at a single base pair. Other techniques, such as 3SR, which utilize RNA polymerase to achieve high copy number, can also be used where appropriate.

In yet another method, PCR may be followed by restriction endonuclease digestion with subsequent analysis of the resultant products. Nucleotide substitutions can result in the gain or loss of specific restriction endonuclease site. The gain or loss of a restriction endonuclease recognition site facilitates the typing of the *C. jejuni* strains associated outbreak using restriction fragment length polymorphism (RFLP) analysis or by detection of the presence or absence of a polymorphic restriction endonuclease site in a PCR product that spans the sequence of interest.

For RFLP analysis, DNA is obtained, for example from the stool of the subject suspected of containing *C. jejuni*, or *C. jejuni* isolated from subject, is digested with a restriction endonuclease, and subsequently separated on the basis of size by agarose gel electrophoresis. The Southern blot technique can then be used to detect, by hybridization with labeled probes, the products of endonuclease digestion. The patterns obtained from the Southern blot can then be compared. Using such an

approach, *PorA* DNA is detected by determining the number of bands detected and comparing this number to the DNA from *C. jejuni* strains that are not associated with the *C. jejuni* outbreak. Restriction endonucleases can also be utilized effectively to detect mutations in the *PorA* gene.

Similar creation of additional restriction sites by nucleotide substitutions at the disclosed mutation sites can be readily calculated by reference to the genetic code and a list of nucleotide sequences recognized by restriction endonucleases.

In general, primers for PCR and LCR are usually about 20 bp in length and the preferable range is from 15-25 bp. Better amplification is obtained when both primers are the same length and with roughly the same nucleotide composition. Denaturation of strands usually takes place at 94°C and extension from the primers is usually at 72°C. The annealing temperature varies according to the sequence under investigation. Examples of reaction times are: 20 mins denaturing; 35 cycles of 2 min, 1 min, 1 min for annealing, extension and denaturation; and finally a 5 min extension step. PCR amplification of specific alleles (PASA) is a rapid method of detecting single-base mutations or polymorphisms. PASA (also known as allele specific amplification) involves amplification with two oligonucleotide primers such that one is allele-specific.

The desired allele is efficiently amplified, while the other allele(s) is poorly amplified because it mismatches with a base at or near the 3' end of the allele-specific primer. Thus, PASA or the related method of PAMSA may be used to specifically amplify the mutation sequences of the invention. Where such amplification is done on *C. jejuni* isolates or samples obtained from an individual during outbreak, it can serve as a method of detecting the presence of the mutations in the strain responsible for the cause of the outbreak.

As mentioned above, a method known as ligase chain reaction tLCR) can be used to successfully detect a single-base substitution. LCR probes may be combined or multiplexed for simultaneously screening for multiple different mutations. Thus, LCR can be particularly useful where, as here, multiple mutations are predictive of the *C. jejuni* strain that is specifically associated with an outbreak.

Antigen-Detecting Kit

The present invention provides a kit for the diagnosis of infection by strains of *C. jejuni*. Particularly, the kit can detect the presence of *PorA* antigen specifically reactive with an antibody or an immunoreactive fragment thereof. The kit can include an antibody bound to a substrate, a secondary antibody reactive with the antigen and a reagent for detecting a reaction of the secondary antibody with the antigen. Such a kit can be an ELISA kit and can comprise the substrate, primary and secondary antibodies when appropriate, and any other necessary reagents such as detectable moieties, enzyme substrates and color reagents as described above.

The diagnostic kit can, alternatively, be an immunoblot kit generally comprising the components and reagents described herein.

Antibody-Detecting Kit

The diagnostic kit of the present invention can be used to detect the presence of a primary antibody specifically reactive with *PorA* or an antigenic fragment thereof. The kit can include the antigen bound to a substrate, a secondary antibody reactive with the antibody specifically reactive with the *PorA* antigen and a reagent for detecting a reaction of the secondary antibody with the primary antibody. Such a kit can be an ELISA kit and can comprise the substrate, antigen, primary and secondary

antibodies when appropriate, and any other necessary reagents such as detectable moieties, enzyme substrates and color reagents as described above. The diagnostic kit can, alternatively, be an immunoblot kit generally comprising the components and reagents described herein.

Nucleic Acid Detection (Diagnostic) Kits

Once the nucleotide sequence of the *PorA* antigen is determined, the diagnostic kit of the present invention can alternatively be constructed to detect nucleotide sequences specific for the antigen comprising the standard kit components such as the substrate and reagents for the detection of nucleic acids. Because *C. jejuni* infection can be diagnosed by detecting nucleic acids specific for the antigen in intestinal tissue and stool, it will be apparent to an artisan that a kit can be constructed that utilizes the nucleic acid detection methods, such as specific nucleic acid probes, primers or restriction fragment length polymorphisms in analyses. It is contemplated that the diagnostic kits will further comprise a positive and negative control test.

The particular reagents and other components included in the diagnostic kits of the present invention can be selected from those available in the art in accord with the specific diagnostic method practiced in the kit. Such kits can be used to detect the antigen in tissue and fluid samples from a subject.

The following examples are intended to illustrate, but not limit, the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

The following Experimental Section discloses the testing and experimentation on which the present invention is based.

EXPERIMENTAL SECTION

EXAMPLE 1

MATERIALS AND METHODS

Bacterial Strains and Media.

C. jejuni, strain 2483, was isolated from a patient with gastroenteritis (23, 24) and was used for the localization and sequencing of the porin gene (*porA*). The organism was passed twice on tryptic soy agar containing 5% sheep blood (TSA) following isolation from the patient and was subsequently stored at -70°C in tryptic soy broth containing 5% sheep blood. Strains of *Campylobacter* sp. and related organisms were maintained at -80°C in glycerol-peptone water as part of the reference collection at the Laboratory Centre for Disease Control, Ottawa, Canada.

Vectorette Polymerase Chain Reaction (PCR).

Genomic DNA from *C. jejuni* strain 2483 was purified as described previously (25). A total of 15 ng of genomic DNA was digested for 2 h at 37°C with 120 U of *Bam*HI, *Eco*RI, *Nhe*I, *Spe*I, *Xba*I, *Hind*III, *Bgl*II and *Bcl*I restriction enzymes (Boehringer Mannheim, Laval, Quebec, Canada). The vectorette oligonucleotides, vectorette universal primer and degenerate primers were synthesized on an Oligo™ 1000M DNA synthesizer (Beckman, Fullerton, Ca.) and are listed in Table 1.

Table 1

Primers used for vectorette PCR and for sequencing *porA* gene in *C. jejuni* strain 2483 porin gene.
R and F are for reverse and forward direction of the primers.

Primer designation	Sequence	SEQ ID NO:
3'-VP	5'-CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATC-GCTGTCCTCTCCTTC-3'	4
5'-*Bam* HI	5'-GATCGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAAC-GGAGGAGAGAAGGGAGAG-3'	5
5'-*Eco* RI	5'-AATTGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACG-GAGGAGAGAAGGGAGAG-3'	6
5'-*Hind* III	5'-AGCTGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAAC-GGAGGAGAGAAGGGAGAG-3'	7
5'-*Nhe* I	5'-CTAGGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAAC-GGAGGAGAGAAGGGAGAG-3'	8
UVP	5'-CGAATCGTAACCGTTCGTACGAGAATCGCT-3'	9
p-1F	5'-GGTAATTTTGATAAAAATTT-3'	10
p-2F	5'-GATACAGGTAAATTTGATAA-3'	11
p-3F	5'-GAAGAAGCTATCAAAGATGT-3'	12
p-4R	5'-TGCCACCATCAACAGCGTTG-3'	13
p-6R	5'-TAAGTAAGCACCTTCAAGTG-3'	14
p-7R	5'-ACTTGTGCTCTATATTTGTG-3'	15
p-10F	5'-TGATAGCGAACTTGATGATA -3'	16
p-13R	5'-AGCATCCCAACCATTTACTT-3'	17
p-14F	5'-TGACTTCGTATATGGTGGAA-3'	18
p-15F	5'-CTCCAAATTTATGTGCTACA-3'	19
p-16R	5'-CTATCAAATTTCCAACTTCT-3'	20
p-17F	5'-TGAAGATGTTGCTACAAGTG-3'	21
p-18R	5'-CTACTCTTGCAACAGCTTCA-3'	22
p-19F	5'-CTTCAAAGCTTTCATTCAGT-3'	23

Common linkers were allowed to anneal as outlined previously (37) by adding 10 mM concentration of each dephosphorylated 57-mer top strand with the 53-mer bottom strand (3'-VP) at 65°C for 2 min followed by cooling to 37°C over the next 20 min. A 30 μl ligation mixture was made with each digest and each contained 2.5 μg of digested genomic DNA, 1 μl of annealed common linkers with the corresponding compatible ends (i.e. *Bam*HI digested genomic DNA with 5'-*Bam*HI-), 1 mM ATP, 10 U T4 DNA ligase (Boehringer Mannheim) and 10 mM DTT and incubated overnight at 15°C.

Polymerase chain reaction of vectorette library and inverse PCR.

Primers p-1F, p-2F and p-3F were generated from the sequenced amino-residues (3, section 3, page XX) number 23 to 29, 21 to 27 and 4 to 10 respectively. Design of the primers was aided by a codon usage chart available through Genebank enabling a degeneracy of 2, 4 and 6 to be obtained for each, respectively. Three separate PCR reaction mixtures were prepared by adding 1 μM of each degenerative primer (Table 1) with 1 μM universal vectorette primer (UVP), 100 μl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1% Triton™ X-100, 30 mM $MgCl_2$), 200 μl of a stock solution containing 200 mM of each dNTP, 20 U Taq DNA polymerase (Promega, Madison, WI). The final volume was raised to 1 ml with sterile ddH_2O. Five microliters of each ligation mixture were added to 50 μl of each of the PCR reaction mixtures followed by amplification in a PE9600 thermocycler (Perkin-Elmer, Foster City, Ca.) with initial melting temperature set at 95°C for 2 min followed by 35 cycles at 95°C for 30s, 55°C for 30s and 72°C for 2 min with a final extension at 72°C for 2 min. The PCR reactions and a 100 bp ladder (Gibco BRL, Grand Island, NY) were electrophoresed on 1% low

melting point agarose (LMP) (Gibco BRL) in 1X TAE buffer and stained with ethidium bromide for 30 min. PCR products were excised from the agarose, extracted using the Promega™ PCR Preps DNA purification system (Promega).

Inverse PCR was performed by first adding 2.5 μg of *Hind* III digested genomic DNA with 6 μl of 100 mM DTT, 6 (l of 10 mM ATP, 5 U of ligase (Boehringer Mannheim), 6 μl of ligase buffer (Boehringer Mannheim) and sterile ddH_2O to give a final volume of 60 μl. The mixture was allowed to ligate overnight at 15°C. Following ligation, PCR was performed as stated above using p-3F and p-7R primers (Table 1) for 35 cycles. The PCR reaction was run on a 1% LMP gel and stained with ethidium bromide. Amplicons were extracted with Promega™ PCR Preps DNA purification system (Promega) and DNA sequenced. The amplicons were DNA sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, Ca) using the Prism™ dye terminator cycle sequencing kit (Applied Biosystems). Analysis of DNA sequences were performed using Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, MI) and PC/Gene (Intelligenetics, Mountain View, Ca.).

Southern blot analysis of genomic DNA.

C. jejuni genomic digests using the restriction enzymes outlined above, were set up and allowed to digest overnight at 37°C. Prior to electrophoresis, *Hind* III digested lambda DNA was labeled with digoxigenin-11-uridine-5'-triphosphate using a random labeling kit (Boehringer Mannheim) for 1 h at 37°C. Digested DNA was electrophoresed on a 1 % agarose gel (Gibco) in 1X TAE buffer together with the *Hind* III digested lambda DNA ladder (Boehringer Mannheim). Following gel electrophoresis, the gel was Southern blotted using established procedures (38) by first placing the gel in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 1 h at room temperature on an orbital shaker followed by 1 h in

neutralizing solution (0.5 Tris-HCl, pH 7.5, 1.5 M NaCl) under the same conditions. The genomic DNA was transferred to Hybond™-N+ nylon membranes (Amersham, Arlington, Heights, IL.) using a Posiblot™ system at 75 mm Hg for 90 min (Stratagene, Aurora, Ontario, Canada).

After transferring, the membrane was washed once with 2X SSC before being UV cross-linked in a UV Stratalinker™ 2400 (Stratagene). Crossed linked membranes were prehybridized at 55°C for 1 h in 10 ml prehybidization solution (Gibco BRL). The PCR amplicon generated from the p-3F and p-6R primers (Table 1) was extracted from a 1% LMP and 10-25 ng was digoxigenin-labeled using a PCR digoxigenin-labeling kit (Boehringer Mannheim) with the same PCR conditions as for the vectorette PCR with exception that 15 cycles were used instead of 35. Approximately 50 ng of lambda ladder probe and the digoxigenin-labeled cytotoxic protein probe were heat denatured at 100°C for 10 min, placed on ice for 5 min and added to the hybridization solution at 55°C overnight.

Following hybridization, the membrane was washed twice for 15 min each in 2X SSC in 0.1% SDS at room temp followed by two 15 min washes first at 55°C in 1X SSC in 0.1% SDS and then 0.1X SSC in0.1% SDS. The washed membrane was blocked in 5% blocking reagent (Boehringer Mannheim) for 1 h on an orbital shaker prior to the addition of anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) used at a dilution of 1:5000 for 1 h. The membrane was washed 3 times for 5 min each in low salt Tris-buffered saline (TBS) and placed in a 1:20 dilution of CPD-Star lumigen substrate (Tropix, Bedford, Ma) in washing buffer containing 0.1M Tris-HCL at pH. 9.5, 0.1 M NaCl and 50 mM $MgCl_2$ for 5 min. The membrane was exposure to high performance autoradiography film (Hyperfilm-MP™) (Amersham) until a suitable band intensity was achieved.

Cloning and DNA sequencing of porin gene.

The region of the genomic DNA digested with *Spe* I which reacted with the cytotoxic probe, was excised from a subsequent LMP gel, and extracted using Geneclean™ (Biol 101, Lajolla, Ca). To determine the optimal concentration of insert to vector, various concentrations of inserts were ligated to 50 ng of *Xba* I digested and alkaline phosphatase (Promega) treated pUC 19 (Pharmacia Biotech, Uppsala, Sweden) with 2.5 U of T4 DNA ligase and 1mM ATP (Promega) at 15°C overnight. A total of 50 ng of vector was used to transform Epicurian coli XL1-blue competent cells as outlined by the manufacturer (Stratagene) and 100 μl was plated to Luria broth (LB) agar plates containing 200 $\mu g/ml$ ampicillin. For color development, plates were covered with 50 μl halogenated indolyl-β-D-galactoside (Bluo-gal) at 20 mg/ml (Gibco BRL) and 15 μl isopropylthio-β-galactoside (IPTG) used at 0.5 M (Gibco BRL). These were allowed to dry prior to the addition of transformants.

Transformants were picked from the plates and grown overnight in 3 ml of LB with 200 $\mu g/ml$ ampicillin. Plasmid preps were performed on 1.5 ml of culture using the Promega™ miniprep DNA purification system (Promega). A total of 50 ng of purified plasmid from the *Spe* I ligation was added to 50 μl PCR mixture containing p-3F and p-6R and amplified for 20 cycles using the same method outlined above. Reactions were electrophoresed on a 1% agarose gel in TAE buffer and stained with ethidium bromide. Plasmids from positive clones were sequenced as described above using the primers given in Table 1. PCR was performed on genomic DNA using primers p-15F and p-16R using the same method as for the porin probe.

Screening of *C. jejuni* isolates for porin gene and cytotoxin production.

A total of 30 strains of *C. jejuni* and related organisms, including strain 2483 (Table 2), were grown on trypic-soy agar containing 10% sheep blood for 48 h in a micro-aerobic environment.

Table 2
Screening of 20 strains of *C. jejuni* for phenotypic expression of a cytotoxin and presence of *porA* using primers specific for the porin gene sequenced from *C. jejuni* strain 2483
(In the Table NT = not tested; ND= not determined)

Organism	LCDC number	Source	Lior serotype	Biotype	Toxin positive	PCR positive
C. jejuni	3454	human	4	Ia	+	+
	3969	ND	untypable	I	+	+
	4951	human	7	I	+	+
	4966	human	7	I	+	+
	6847	human	1	Ia	+	+
	7099	chicken	61		+	+
	7288	water	9	II	+	+
	8916	human	94	IIa	+	+
	9214	human	2	Ia	+	+
	9541	water	82	II	+	+
	9543	water	82	II	+	+
	9555	human	23	I	+	+
	10403	human	36	Ia	+	-
	10673	human	82	II	+	+
	14040	human	82	II	+	+
	14906	human	82	I	+	+
	15151	human	82	I	+	+
	16323	beef	82	I	+	+
	16334	human	82	II	+	+
	16336	human	82	II	+	+
	16388 (2483)	human	82	II	+	+
	1	ND	4	I	+	+
	2074	ND	36	II	+	-
C. Lari	729	ND	31	I	+	-
C. coli	348	ND	14	I	+	-
C. sputorum subsp. *fecalis*	5754	ND	NT	NT	+	-
C. fetus subsp. *fetus*	7055	ND	NT	NT	+	-
C. hyointestinalis	8494	human	NT	NT	+	-
C. jejuni subsp. *doylei*	9365	ND	NT	NT	+	-
A. butzleri	13220	human	7	IIIA	+	-
E. coli VT1+		ND	NT	NT	+	-
E. coli VT2+	1119	human	NT	NT	+	-

Using a bacteriologic sample loop of each strain were removed and the DNA extracted as previously described (25). PCR conditions were as stated above except 50 ng of genomic DNA was used in the reaction with p-3F and p-6R for 35 cycles at an annealing temperature of 55°C. The PCR reactions were then mixed with 6X sample buffer and 20 μl of each were electrophoresed on a 1% agarose gel in TAE buffer and stained with ethidium bromide. Each strain was also screened for phenotypic expression of a cytotoxin in a biphasic system using a 12-well cell culture plate (Costar). A loop of each strain was inoculated into 2 ml of minimal essentail media (MEM) without fetal bovine serum (FBS) and used to overlay 1 ml of Meuller-Hinton agar present in the bottom of the wells. The organisms were grown for 48 h at which time the liquid media was removed and centrifuged to remove the bacteria. HEp-2 cells were subcultured into 96 well plates at a density of 1 X 10^4 cells/well with 200 μl MEM supplemented with 10 % FBS 24 h prior to the addition of the toxic filtrate. The supernatant was assayed for cytotoxic activity by replacing the 200 μl of growth media used in subculturing the Hep-2 cells with 200 μl of the organisms free filtrate. The cells were monitored over a 48 h period for cytological changes. *E. coli* O157:H7 strain 3787 (H19), positive for verotoxin type 1 (VT1), and strain 90-2380, positive for verotoxin type 2 (VT2), were used as postive controls for the cell culture assay while uninoculated media was used as the negative control.

Genebank accession number.

Results

Vectorette PCR.

Vectorette PCR was performed using the genomic DNA digested with *Nhe*I ligated to its corresponding common oligonucleotide and these generated amplicons suitable for

DNA sequencing. The universal primer (UVP) and p-1F, p-2F and p-3F yielded amplicons of similar size of approximately 800 bp in length which was consistent with the position of the *Nhe* I restriction site (Fig 1) and the position of the primers (Table 1). DNA sequencing of the three amplicons revealed the same sequence which, when translated, contained an ORF corresponding to the protein sequence obtained from the N-terminus of the cytotoxin. No other amplicons were seen with the remaining genomic digests when the PCR conditions were maintained. From the DNA sequence, primer p-6R was designed from nucleic acid positions 768 to 749 of the sequenced amplicon. The new primer, along with p-3F were subsequently used to amplify the 650 bp probe used for Southern blot analysis and localization of the cytotoxic porin gene.

Partial cloning and sequencing of cytotoxic porin protein.

Southern blot analysis of the digested genomic DNA using the digoxigenin-labeled cytotoxic porin probe yielded several discrete bands when probed with the 650 BP probe (Fig 2). The *Spe* I fragment was chosen, purified and ligated to pUC 19 and used to transform Epicurian coli XL1-blue competent cells. One colony from the *Spe* I digested and extracted genomic DNA, which was positive by PCR for the 650 bp product was designated Cj08 and this was sequenced.

Inverse PCR was only performed on *Hind* III digested DNA because of the results obtained from the Southern blot analysis using the 650 bp probe and the restriction map (Fig 2) of the amplicon initially generated from the *Nhe* I vectorette library. The Southern blot showed a weak reaction between the digoxigenin-labeled probe and an approximate 800 bp fragment making it a potential candidate for amplification by inverse PCR. A new primer, p-7R, which was generated from positions 5'-209→190-3' of

the sequenced amplicon from the vectorette PCR, together with p-3F produced an 800 bp product with the ligated *Hind* III digested genomic DNA. When sequenced the amplicon was found to contain the N-terminus of the porin protein along with the entire leader sequence with the start codon and the ribosome binding site. The sequence data and translated protein obtained from the clone and the inverse PCR are shown in Figure 3. The restriction map revealed a *Spe* I restriction site at position 6 of the function gene; therefore the *Spe* I clone only contained part of the functional gene, but the remainder of the gene was elucidated from the sequence the amplicon generated from the inverse PCR reaction.

The entire gene was found to be 1275 bp in length [SEQ ID NO:3] and was designated *porA*. The protein encoded was 424 amino acids in length (Fig 3) [SEQ ID NO:2] and had a calculated molecular weight of 45.6 kDa and a pI of 4.44. The leader sequence was found to be 22 amino acid residues in length and was cleaved from the active protein conforming to the -3,-1 rule (2) between the Ala→22 (A) and Thr→23 (T), which was the first amino acid residue in the sequenced protein. The mature protein, minus the leader sequence has a calculated molecular weight of 43.5 kDa and had a pI of 4.35. These finding were consistent with previous reports (3,18, 21) regarding the size and pI of the porin protein from *C. jejuni*. Primers, p-15F and p-16R were subsequently designed to PCR amplify the entire porin gene (Fig. 4) as well as for use in the sequencing reactions.

Sequence analysis.

Sequence homology searches were performed on the entire ORF and translated protein using GCG (Genetics Computer Group, Madison, WI). The translated porin protein from *C. jejuni* strain 2483 had no significant homology with any characterized protein except with the

previously described *C. jejuni* porin protein (3) and the 45 kDa and 51 kDa outer membrane proteins form *Wolinella recta* (20). However, the DNA sequence had the greatest similarity with *H. influenzae* outer membrane protein P2 over short stretches following a BLAST data base search (BLAST; Beckman Center for Molecular and Genetic Medicine, Stanford University of Medicine). A comparative analysis of the translated porin from *C. jejuni* strain 2483 and several bacterial outer membrane proteins revealed that *C. jejuni* porin protein had a 50% sequence similarity but only 23% sequence identity with the *H. influenzae* major outer membrane protein P2. In addition, in those regions of the DNA where the homology was greatest, the protein sequence identity was as much as 72%. The porin from *C. jejuni* also had a 46% similarity and 21% identity with the *Enterobacter cloacae* pore forming outer membrane protein PhoE, 44% similarity and 21% identity with *Klebsiella pneumoniae* PhoE, 43% similarity and 17% identity with *Salmonella typhi* ompC, and 42% similarity and 19% identity with *E. coli* PhoE. When the porin from *C. jejuni* was compared to the consensus sequence obtained from an alignment of ompF, ompC, PhoE and Lc of *E. coli*, PhoE of *K. pneumoniae* and ompC of *S. typhi* (29), a 45% similarity and 20% identity was found.

Screening *Campylobacter* sp. for *porA* and cytotoxin production.

Results of screening *C. jejuni* for phenotypic and genotypic expression of the cytotoxin gene are summarized in Table 2. It was found that all 32 strains of *Campylobacter* sp. and related organisms produced a cytotoxic component when the filtrate from the biphasic

growth system was assayed in tissue culture but only 22 of 32 (69%) were PCR positive using the primers (p-3F and p-6R) specific for *porA*. However, 19 of 20 (95%) of the *C. jejuni* strains screened for *porA* were PCR positive for the 650 bp product showing that the porin gene was highly conserved among strains of *C. jejuni*, especially Lior serotype 82, but was not conserved between related species of *Campylobacter*.

DISCUSSION

The 1275 bp ORF had a %guanosine+cytosine content of 36.8 mol % (Fig. 3) which is slightly higher than the range previously described for *C. jejuni* chromosomal DNA (42, 43). A putative Shine-Dalgarno (SD) sequence which has previously been described (33) with a sequence of 5'-AGGAG-3', lies centered 10 bp upstream for the initiation codon ATG. A putative -35 region, which has been previously described (33) is centered 87 bp upstream from the initiation codon with a sequence of 5'-TTTACT-3' while a putative -10 region, 5'-TTAAGA-3' is centered 57 bp upstream (Fig 3). Both the -35 and -10 sequences were predicted as putative sites using PC/Gene software package (Intelligenetics, Inc.) and comparative analysis with putative sites from published sequences from *C. jejuni*. A potential termination sequence does existed 25 bp down stream from the stop codon 5'-TAA-3' (Fig 5). It consisted of a 9 bp dyad stem loop with a predicted stability of -19.2 kcal/mol separated by 5 unpaired bases which could comprise the loop structure. This is followed by a poly-T region and could signify a rho-independent termination sequence (1).

With very few exceptions, codon usage in the coding region of *porA* gene are consistent with the compilation available through Genebank (Table 3 below).

Table 3
Codon usage chart for the 1275 bp open reading frame *porA* from *C. jejuni* strain 2483.

	UAA	-	Ile	AUC	9	Arg	CGU	1
	UAG	-		AUA	2		AGA	7
	UGA	-		AUU	4		CGC	1
Ala	GCU	35	Lys	AAA	22		CGA	0
	GCC	1		AAG	0		AGG	0
	GCG	1	Leu	CUU	17		CGG	0
	GCA	11		CUA	4	Ser	UCA	6
Cys	UGU	0		CUG	0		UCC	0
	UGC	0		UUG	0		UCG	0
Asp	GAU	30		UUA	17		UCU	6
	GAC	2		CUC	0		AGC	7
Glu	GAG	2	Met	AUG	2		AGU	6
	GAA	15	Asn	AAC	19	Thr	ACG	1
Phe	UUU	10		AAU	15		ACC	0
Gly	GGC	3	Pro	CCC	0		ACU	14
	GGA	9		CCU	1		ACA	14
	GGG	0		CCA	3	Val	GUC	1
	GGU	32		CCG	0		GUG	2
His	CAC	2	Gln	CAG	2		GUU	12
	CAU	2		CAA	13		GUA	20
Trp	UGG	5						
Tyr	UAC	11						
	UAU	12						

For instance, Tyr was equally encoded by UAU and UAC while GUA was used more frequently to encode Val rather than GUU and AUC encoded Ile instead of AUU. The most striking difference was the number of Phe encoded by UUC which had previously been shown to be encoded more frequently by UUU while AAC rather than AAU encoded Asn. These frequencies were most likely due to the quantity of G+C residues in the coding region and these may confer an increase in gene stability at increased temperatures especially as the organism is considered to be thermophilic.

The ORF [SEQ ID NO: 3] was found to produce a 45.6 kDa protein with a pI of 4.44 both of which are consistent

with previous reports on the *C. jejuni* porin protein (3, 18, 21). The translated protein was also found to contain several hydrophobic regions as determined by the method of Novotny and Auffray (31) (PC/Gene). Structural prediction using the method of Garnier (12) (PC/Gene) and Novotny (31) indicated there was also considerable secondary structure associated with the porin with 50% of the amino acids forming extended or β-pleated sheets conformations. This was consistent with previous circular dichroism (CD) findings (3) as well as with other bacterial porin proteins (29). The number of residues necessary to span the membrane has been estimated from *Rhodobacter capsulatus* porin to be between 6 to 17 residues in length (35). Based on this assumption, together with the β-pleated sheets diagram and hydrophobic chart, there may be as many as 12 membrane spanning domains while the enterobacterial consensus sequence (29) and *R. capsulatus* both contain 8β-strands (29).

The relative amount of sequence identity was low compared to the sequence similarity. This indicated that although the primary structure was quite distinct, the properties associated with the 424 amino acid protein were similar to those of other well characterized porins. For instance, the relative amounts of basic, polar and acidic residues are similar to that of *H. influenzae* P2 as well as the enterobacterial consensus sequence; however, there was a greater frequency of hydrophobic residues in the porin from *C. jejuni* (Table 4).

Table 4

Comparison of the amino acid composition of *porA* from *C. jejuni* strain 2483, *H. influenzae* P2 and the consensus sequence from enterobacterial porin. Numbers in parenthesis represent residues in the leader sequence.

	No. Residues/mol in:		
Amino Acid Group	*C. jejuni* por A	*H. influenza* P2[a]	Consensus Enterobacterial porin[b]
Basic			
Lys	22 (2)	30 (2)	23
Arg	9	16	11
His	4	7	1
Acidic			
Asp	32	17	34
Glu	17	24	17
Polar			
Asn	34 (1)	25 (1)	24
Cys	0	0	0
Gln	15	14	17
Gly	44 (1)	40 (1)	40
Ser	25 (2)	17 (1)	18
Thy	29	24 (1)	22
Tyr	23	23	23
Hydrophobic			
Ala	48 (8)	24 (8)	26
Ile	15	15 (1)	9
Leu	38 (4)	24 (2)	23
Met	2 (1)	1 (1)	5
Phe	23 (1)	13 (1)	21
Pro	4	3	4
Trp	5	0	3
Val	35 (2)	24 (1)	15

[a] Data derived from Hansen et al. (17)
[b] Data derived from H. Nikaido (29)

This could coincide with more membrane spanning regions leading to a more extensive secondary structure. When the *C. jejuni* porin sequence was compared to the hypothetical folding pattern of the enterobacterial consensus sequence, there was no more significant similarities in the membrane spanning regions of the consensus sequence than in the remaining sections. Comparison of the N-terminal sequences of *H. pylori* porin proteins (9, 10) showed very little sequence identity with the porin from *C. jejuni*. However, when the sequences were compared for similarities, the porin from *C. jejuni* had the greatest similarity with HopC (57%) followed by HopE (55%) then HopD and HopB (50%) and the least similarity with HopA (47%). The conductance levels of the channels formed by the *H. pylori* porin range from 0.25 to 0.36 nS (10) which is considerably lower than the conductance of 8.82 nS reported for the *C. jejuni* porin (18). The molecular weights of each of the *H. pylori* porins are greater than the porin from *C. jejuni* and also appear to present as monomers in lipid bilayers (10) instead of the trimeric form similar to *C. jejuni* porin (3).

The MOMP has been found to elicit an immune response both in humans (30) and rabbits (11) making it a suitable candidate for vaccine development. PCR studies to determine the frequency of the porin gene in other strains of *C. jejuni* showed that 95% of these contained at least

part, if not all of the intact gene while the other *Campylobacter* sp. and related organism were PCR negative. Previous reports indicated that only 60% of pathogenic strains possessed a protein of similar size as determined by SDS-PAGE and Western blot analysis using antiserum against the MOMP from *C. jejuni* strain 85H (21) (a sample of which was deposited at the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852 USA on March 19, 1998 under the terms of the Budapest Treaty, accession no. ATCC 202,102). The PCR results outlined above are valuable, and provide a new and efficient method to identify *C. jejuni* from other *Campylobacter* sp. The potential for the development of a recombinant vaccine using the porin protein is also noteworthy. Previous studies by Gonzales *et al.*(13) have shown that T-cell activation occurred through the induction of lymhokines by *S. typhi* porin and, as a consequence, this may play a role in protective immunity. Protection in guinea pigs was seen by using the enterobacterial outer membrane protein PhoE as a vector to express B-cell epitopes on the surface of *E. coli* providing a vehicle for live vaccine development (45).

EXAMPLE 2

MATERIALS AND METHODS

Bacterial strains and culture media

C. jejuni strain 2483 was isolated from a patient with gastroenteritis and was characterized as Lior serotype 82, biotype 1 and Penner serotype O:11. The organism was passed twice on tryptic soy agar containing 5% sheep blood (TSA) following isolation from the patient and was subsequently stored at -70°C in tryptic soy broth containing 5% sheep blood. Thawed aliquots were cultured

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on TSA with 5% sheep blood prior to inoculation into Brucella broth (BBL, Cockeysville, MD, USA) pre-equilibrated in an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2. For batch preparation, a suspension of the

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organism was made equivalent in density to a McFarland number 8 standard and inoculated into 4 L of Brucella broth at a density of 2 ml/L. Inoculated broths were incubated under stationary conditions in the gas mixture for 48 h at 37°C.

Isolation of cytotoxic complex

Bacteria were harvested by centrifugation at 12,000 x g for 20 min at 4°C and the 4 L of organism-free filtrate were concentrated by ultrafiltration at 4°C with a stirred cell apparatus using a YM30 membrane (30,000 NMWL) (Amicon, Beverly, MA, USA). The filtrate was initially concentrated approximately 40-fold by ultrafiltration and further by the addition of ammonium sulfate to 80% saturation at 4°C. The ammonium sulfate precipitated proteins were collected by centrifugation at 12,000 x g for 30 min and resuspended in 50 mM Tris-HCL buffer, pH 7.0. Purification of the cytotoxic protein was performed using a Hewlett Packard 1050 series high performance liquid chromatograph (HPLC) equipped with a diode-array detector. Purification was initiated by adding concentrated filtrate at 1% of the total bed volume to a HiLoad™ 16/60 Superdex™ 75 sizing column (Pharmacia Biotech, Uppsala, Sweden) and eluting with phosphate buffered saline, pH 7.0 (PBS) at a flow rate of 1 ml/min. Fractions were collected on a Gilson fraction collector and 50 μl of each was evaluated for cytotoxic activity using HEp-2, HeLa and CHO cells. The molecular mass of the native cytotoxic complex was determined by calibrating the column using low molecular weight standards (Pharmacia Biotech) dissolved in PBS. Cytotoxic-containing fractions were pooled, concentrated using Centraprep-30 units (Amicon), and applied to a 7.5 X 75 mm TSK DEAE-5PW column (Pharmacia Biotech). Proteins were eluted using a linear gradient of 0.2-0.25 M NaCl in 50 mM Tris-HCl, pH 7.0 at a flow rate of 1 ml/min. Fractions were collected, desalted by spin dialysis using Centricon-30 units (Amicon) and 50

μl of each sample was applied to monolayers of HEp-2, HeLa and CHO cells for assessment of cytotoxic activity.

The bacterial pellet removed from the filtrate was placed on ice and sonicated using a Bronson Sonifier 450™ sonicator (Branson Ultrasonic Corporation, Danbury, CT), centrifuged at 12,000 X g for 10 min and the supernatant assayed in the same manner as the filtrate.

Polymerase chain reaction

Genomic DNA was isolated from *C. jejuni* strain 2483 by standard procedures (70). Polymerase chain reaction (PCR) was conducted as outlined previously (71) using 50 ng of genomic DNA with *E. coli* verotoxin VT1a primers (GAAGAGTCCGTGGGATTACG) [SEQ ID NO:24] and VT1b (AGCGATGCAGCTATTAATAA) [SEQ ID NO:25] and VT2a (TTAACCACACCCACGGCAGT) [SEQ ID NO:26] and VT2b (GCTCTGGATGCATCTCTGGT) [SEQ ID NO:27] at 42°C, 45°C, and 50°C annealing temperatures. PCR was also conducted using primers DZ3 (AGTAAGGAGAAACAATGA) [SEQ ID NO:28] and R009 (AATAAGCCTTAGAGTCTTTTTGGAATCC) [SEQ ID NO:29] specific for *Helicobacter pylori cagA* and primers F6 (GCTTCTCTTACCACCAATGC) [SEQ ID NO:30] and R20 (TGTCAGGGTTGTTCACCATG) [SEQ ID NO:31] specific for *H. pylori vacA* gene as outlined previously (72). The *H. pylori* Penner reference serotypes 05 and 06 were used in PCR reactions as positive controls for the *cagA* and *vacA* respectively. The PCR methodology used was as described for *cagA* (72) except that 100 ng/reaction of chromosomal DNA was used for amplification of the *vacA* gene using 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min with a final extension of 10 min. PCR reactions were electrophoresed on a 1% agarose gel in Tris-acetate, EDTA-containing buffer (pH 8.3), stained with ethidium bromide and visualized on a transilluminator

(Ultra-violet Products, Inc, San Gabriel, CA, USA).

Cytotoxic activity and protein determination

The amount of protein at each stage of the isolation procedure was quantified using the BCA protein assay (Pierce, Rockford, IL, USA). HEp-2, HeLa and CHO cells were grown in T-75 cell culture flasks (Costar, Cambridge, MA) using Eagle's minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA). Cells were subcultured into 96-well plates 24 h prior to determination of cytotoxic activity. Cytotoxic activity was quantified in the three cell lines as described previously (54) and activities expressed after 48 h incubation as tissue culture dose 50 (TCD_{50}). A TCD_{50} was defined as the amount of toxin required to cause cytotoxic changes in 50% of the cells. Cell cultures were fixed for 10 min in absolute methanol and stained for 30 min with Giemsa™ (Gibco BRL, Grand Island, NY, USA). The specific activities were determined at each step of the isolation and expressed as $TCD_{50}/\mu g$ of protein. *E. coli* O157:H7 strain LCDC 3787 (H19), positive for VT1, and strain LCDC 90-2380, positive for VT2, were used as controls for TCD_{50} determination in HEp-2 and HeLa cells. *V. cholerae* 01, strain 755, an enterotoxin-producing isolate, was used as a control in the CHO cell assay.

Molecular weight and physical characterization of the cytotoxic complex

One μg of the isolated cytotoxic material was mixed with equal volumes of 2X sample buffer containing ß-mercaptoethanol and sodium dodecyl-sulphate. The sample was boiled for 5 min and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% homogeneous gel along with low molecular weight standards and silver stained using a commercial kit (BioRad, Hercules, CA, USA). One μg aliquots of the isolated cytotoxic material were either heated at 70°C for 30 min or treated with trypsin in PBS at concentrations

ranging from 0.03 to 1.25% for 2 h at 37°C. Residual trypsin activity was inactivated by addition of FBS to give a final concentration of 20% for 1 h at 37°C. Heated and trypsin-treated samples were serially diluted 2-fold in PBS prior to cell culture assay to determine the degree of activity remaining after the treatments. Heat inactivated trypsin and FBS alone were used as negative controls.

N-terminal sequencing of cytotoxin

The cytotoxic component was isolated and denatured with SDS and ß-mercaptoethanol and electrophoresed along with kaleidoscope prestained molecular weight standards (BioRad) on a 12% gel SDS-PAGE. Following electrophoresis, the protein and standards were electrophoretically transferred to polyvinylidene diflouride (PVDF) (BioRad) for 18 h at 100 mA in 10 mM 3-[cyclohexylamino]- 1-propanesulfonic acid (CAPS) (Sigma) buffer, pH 11.0 containing 10% methanol. Following transfer, the blot was stained with 0.1% Coomassie blue R-250 (BioRad) in 50% methanol for 5 min and destained with 50% methanol and 10% acetic acid. The immobilized cytotoxic protein was excised from the PVDF and sequenced by Edman degradation on an Applied Biosystems model 473A protein sequencer (CHUL Research Center, Saint-Foy, Quebec, Canada). Protein analysis was performed using Lasergene (DNAStar, Madison, WI, USA).

Neutralization and Western blot analysis

Neutralization studies were performed on 1 μg aliquots of the isolated complex using polyclonal antisera raised against the cytotoxic complex from *C. jejuni*, as well as against *E. coli* VT1, *E. coli* VT2, CDT from *C. jejuni* (54) and CDT from *E. coli* (54), enterotoxin from *V. cholerae* (Sigma) and the cytotoxin from *C. difficile* (Techlab, Blacksburg, VA, USA). Normal rabbit serum was

used as a negative control. Homologous antiserum was raised by intramuscular inoculation of New Zealand white rabbits with 0.5 ml of a 5 μg/ml preparation of isolated cytotoxic material emulsified in 0.5 ml Freund's incomplete adjuvant (FIA). This was followed at weekly intervals for 4 weeks by subcutaneous injection of the same antigen preparation in FIA. A 1:10 dilution of each antiserum was added to serial two fold dilutions of 1 μg of the isolated protein. After a 1 h incubation at 37°C, aliquots of each were added to HEp-2 cells and incubated for 48 h at 37°C. Each antiserum was also assayed using Western blot analysis. *C. jejuni* cytotoxic complex, *E. coli* VT1 and VT2, *C. jejuni* CDT, *E. coli* CDT, *C. difficile* cytotoxin and *V. cholera* enterotoxin were each separated on SDS-PAGE gels along with kaleidoscope prestained molecular weight standards (BioRad) and transferred to 0.2 μm pore size PVDF membranes for 18 h at 100 mA. Membranes were washed with 5% skim milk for 1 h to prevent nonspecific binding of the antibodies and then washed 3 times for 5 min each with PBS. A 1:500 dilution of each of the antisera in a 1% skim milk solution containing 0.05% Tween™-20 was prepared and added to the membranes for 2 h at room temperature. This was followed by a 1 h treatment with 200 mU/ml of goat anti-rabbit alkaline phosphatase conjugated antibody (Boehringer Mannheim, Laval, Quebec, Canada) at room temperature. Membranes were developed using 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Boehringer Mannheim).

Western blot analysis was also performed using crude concentrated filtrates from *C. jejuni* strain 2483, *C. jejuni* LCDC 3969, *C. jejuni* LCDC 16336, *C. coli* strain 8682, *Aeromonas veronii* LCDC A2297 (negative control) and *E. coli* 3787 (positive control for VT1). A total of 40 μg of each crude filtrate was electrophoresed and transferred to PVDF as stated previously and probed with anti-

cytotoxic complex from *C. jejuni*. In addition, each filtrate was tested for cytotoxic activity in HEp-2 cells after 48 h of incubation.

Carbohydrate characterization

A total of 2 μg of the isolated cytotoxic material was assayed for the presence of lipopolysaccharide by the *Limulus* amebocyte lysate (LAL) test according to the package insert (Pyrotell, Associates of Cape Cod, Inc., MA, USA). The toxic material along with *E.coli* LPS were each diluted 10-fold with pyrogen free water in duplicate and 100 μl of each dilution were incubated with 100 μl of Pyortell™ in a 37°C water bath for 1 h. Tubes were inverted and those containing a solid clot were considered positive. To determine whether the lipopolysaccharide contributed the activity of the toxin, 1 μg of the isolated cytotoxic material was incubated for 1 h at 37°C with 5 U neuraminidase (Sigma) at pH 5.0, 3 U of N-glycosidase F (Boehringer Mannheim) at pH 7.2 and 10 mM sodium metaperiodate (Sigma) for 90 min at room temperature. The residual cytotoxic activity was then assayed in HEp-2 and HeLa cells using serial twofold dilutions.

Identification of the carbohydrate moiety was made using a glycan differentiation kit (Boehringer Mannheim) containing five unique digoxigenin-labeled lectins (Table 5). Approximately 1 μg of the isolated cytotoxic protein and 5 μg of each carbohydrate standard were spotted on PVDF membranes and allowed to dry overnight at 37°C. Membranes were probed for the co-purifying LPS according to the manufacturer's insert instructions. Those lectins that gave positive results were further examined by Western blot analysis using 15 μg of each carbohydrate standard and 8 μg of the test carbohydrate from the purified preparation. The isolated cytotoxic material from three separate batches was assayed in μg for

the carbohydrate concentration using a phenol-sulphuric acid assay measured at 490 nm (73). This was expressed as a ratio to the number of μg of purified carbohydrate per μg of purified protein. SDS-PAGE and native PAGE were performed using 10 μg of the carbohydrate and gels were double stained, first with periodic acid-Schiff (PAS) (74) then with Coomassie blue.

Table 5
Specificities and the reactions of the lectins used in the carbohydrate determination

Lectins	Specificity (linkage)	Reactivity*
Galanthus nivalis agglutinin (GNA)	Manα(1-3), α(1-6) or α(1-2)-Man (terminally linked mannose)	+++
Maackia amurensis agglutinin (MAA)	Neu5Acα(2-3)-Gal (sialic acid terminally linked α(2-3) to galactose)	+
Datura stramonium agglutinin (DSA)	Galβ(1-4)GlcNac (galactose-β(1-4)-N-acetylglucosamine)	+
Arachis hypogaea (peanut) agglutinin (PNA)	Galβ(1-3)GalNac (galactose-β(1-3)-N-acetylgalactosamine)	-
Sambucus nigra agglutinin (SNA)	Neu5Acα(2-6)-Gal or GalNac (sialic acid terminally linked α(2-6) to galactose or N-acetylgalactosamine)	-

*+++ strong positive result; + weak positive result; - negative result

Results

Identification and molecular characterization of cytotoxic complex

Cytological signs of intoxication caused by the cytotoxic complex included the formation of vacuoles in the cytoplasm of HEp-2 cells as compared with normal unaffected cells (Fig. 6a and 6b). Similar results were found with HeLa cells. The number of vacuoles in each cell ranged from 1 to 5 with 50% or more of the cytoplasm

of some cells being affected. After 24 h, the vacuoles diminished in size and the cells developed a rounded, highly refractile appearance. By 48 h, cytoplasmic blebbing and nuclear condensation became more evident along with cell loss from the monolayer (Fig. 1c and inset). Toxicity was dose-dependent and was detected using 2-fold serial dilutions of the isolated material. At the lower cytotoxin concentration of 1 μg of protein/well, the vacuoles persisted up to 48 h while rounding occurred up to 72 h. When higher cytotoxin concentrations of 10 μg protein/well were used; vacuoles formed and dissipated within the first 12 h following intoxication and greater than 50% of the cells were rounded and refractile by 24 h. By 48 h, 80-100% of the cells had become rounded (Fig. 6c). Similar cytological changes were observed in all of the cell lines when the whole bacterial cell sonicate was assayed for toxicity. Strain 2483 produced low levels of CDT in the crude concentrate; however, this was neutralizable with polyclonal antisera raised against either *C. jejuni* or *E. coli* CDT (data not shown).

The organisms were grown for 48 h at 37°C in Brucella broth at which time the bacteria were in the stationary phase of growth. Concentrated proteins from the culture supernatant possessing high levels of cytotoxic activity were found to elute from the G75 column in the void volume with a calculated native molecular mass of greater than 100 kDa (Fig 7a). This peak (peak A) was collected and applied to the TSK DEAE-5PW column. Cytotoxic activities of the TSK DEAE-5PW fractions showed the toxin eluted at approximately 0.21-0.22 M NaCl (Fig 7b). The two-column purification procedure produced a single silver-stained protein with a molecular size of 45 kDa calculated by R*f* under denaturing conditions. The toxic activities at each stage of the purification procedure are shown in Table 6. The isolated cytotoxic complex demonstrated highest toxic activity for HEp-2 cells and the lowest for CHO cells.

The cytotoxin was inactivated by heat treatment at 70°C for 30 min but was resistant to trypsin at the concentrations tested.

Table 6

Specific activity expressed as $TCD_{50}/\mu g$ of protein at each step of the purification in Hep-2, HeLa and CHO cells.

Toxin Preparation	Cell Culture Specific Activity*		
	Hep-2	HeLa	CHO
C. jejuni Strain 2483			
crude concentrate	1.56	0.51	0.51
Superdex 75 16/60	1.61	3.88	0.97
TSK DEAE-5PW	20.1	7.49	1.87
E. coli VT1+ Strain LCDC 3787 (H19)	0.35	0.17	NA
E. coli VT2+ Strain LCDC 90-2380	1.48	2.89	NA
V. cholerae O1, Strain 755	NA	NA	0.48

*$TCD_{50}/\mu g$ of protein; NA=no activity

Polymerase chain reaction

Oligonucleotide primers specific for *E. coli* VT1 and VT2 failed to produce amplicons corresponding to A- and B-subunits of mature verotoxin types 1 and 2. Also, primers specific for the *cagA* and *vacA* genes of *H. pylori* failed to generate amplicons.

Protein Alignment

The cytotoxic protein consisted of a single protein with a calculated molecular mass of 45 kDa. The excised band was subjected to N-terminal sequencing and a total of 31 amino acid resides were elucidated (Table 7). The protein was found to contain several hydrophobic and charged residues and had a predicted isoelectric point of

4.35. The protein had 97% homology with the major outer membrane protein (MOMP) from *C. jejuni* which has been characterized as a porin (68) and the single amino acid difference at residue 30 was conserved. The cytotoxic porin also shared 56% and 63% sequence homology with 45 kDa and 51 Kda outer membrane proteins respectively from *Wolinella recta* (75).

Table 7

Sequence homologies of the *C. jejuni* 2483 cytotoxic porin and related sequences as ascertained by BLAST[1] searches

Protein designation	Sequence[2]
C. jejuni cytotoxic porin protein	TPLEEAIKDVDVSGVLRYRYDTGNFDKNFVN
C. jejuni MOMP; porin protein	TPLEEAIKDVDVSGVLRYRYDTGNFDKNF*N
W. recta 45 kDa outer membrane protein	TPLEEAIKDVD-SG---XY-*---X-N--
W. recta 51 kDa outer membrane protein	TPLEEAIK*VD*SG--XYXY*----KN--

[1] Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine.

[2] Capital letters represent identical residues; "*" represent conserved changes; "-" represents mismatch in sequences; "X" represents unknown residue.

Neutralization and Western blot analysis

Polyclonal antisera raised against *E. coli* VT 1 and VT 2, *C. jejuni* and *E. coli* CDT, *V. cholerae* enterotoxin and *C. difficile* cytotoxin failed to neutralize the cytotoxic effects elicited by the *C. jejuni* toxic complex in cell culture. However, when this cytotoxic complex was serially diluted, incubated with rabbit polyclonal antiserum raised against the cytotoxic protein and added to HEp-2 cells, the TCD_{50} occurred at a dilution of 1:2 whereas that of the normal rabbit serum was at a dilution

of 1:32. Neutralization was defined as a decrease in the TCD_{50} at 24 h post-intoxication. Antiserum raised against the cytotoxic protein showed immunological reactivity in Western blots with the purified 45 kDa cytotoxic protein. Antisera raised to the other toxins showed no cross reactivity with either the cytotoxin or carbohydrate on immuno-blot analysis. Western blots of crude concentrated filtrates from various cytotoxic strains of *Campylobacter* species showed the presence of a protein with a molecular mass similar to that of the porin (Fig. 8) while no bands were detected in the blots from the uninoculated broth, and filtrates from *A. veronii* and *E. coli* VT1 strains.

Lipopolysaccharide identification and carbohydrate analysis

The isolated cytotoxic material and *E.coli* LPS were assayed for the presence of endotoxin by incubating serial dilutions with *Limulus* amebocyte lysate for 1 h at 37°C. The cytotoxic material produced a strong positive result at a dilution of 1:128,000 signifying that the isolated cytotoxic material contained LPS. The *E.coli* LPS also gave a positive result. To determine whether or not the cytotoxic activity associated with the complex reside in the protein component, the complex was incubated with 10 mM sodium metaperiodate, to oxidize the free hydroxyl groups present on visceral hexoses, with 5 U neuraminidase, to cleave sialic acid residues and with 3 U N-glycosidase F to cleave asparagine bound N-glycans. The complex was then assayed for cytotoxic activity in HEp-2 and HeLa cells and expressed as TCD_{50} endpoints. Titers of 32 were observed in the HEp-2 cells while a titer of 8 was found in the HeLa cells as well as for the control cells inoculated with untreated toxin.

The carbohydrate component of the LPS was characterized using digoxigenin-labeled lectins (Table 5) and the data revealed a complex of different subunits. Lectin *Galanthus nivalis* agglutinin (GNA) reacted strongly with the purified material and suggested a high proportion of terminally-linked mannose. The lectins *Maackia amurensis* agglutinin (MAA) and *Datura stramonium* agglutinin (DSA) also gave positive but weaker results, indicating the presence of sialic acid terminally linked α(2-3) galactose and galactose-ß(1-4)-N- acetylglucosamine in the complex as well as hybrid N-glycan structures. The remaining lectins showed no reactivity for the carbohydrate complex. The proportion of carbohydrate to protein in the purified material was calculated at a ratio of 4:1. PAS staining revealed a high molecular weight carbohydrate which did not appear as a discrete band as did the protein component of the complex but, instead, occupied a broad range of sizes (Fig. 9). Double staining of purified cytotoxin in native PAGE gels showed no protein component in contrast to samples boiled in denaturing buffer prior to gel electrophoresis (Fig. 9). Western blots performed with the lectins (Fig. 10) showed that the high molecular mass smear seen following PAS staining (Fig. 9) was carbohydrate in nature with high reactivity for GNA (Fig. 10).

Discussion

A cytotoxin from strains of *C. jejuni* that was heat-labile, trypsin-sensitive and induced characteristic rounding of HEp-2, HeLa and MRC-5 cells was first documented by Yeen *et al.* (76). Guerrant et *al.* (58) also described a cytotoxic component which was heat labile at 60°C, was partially sensitive to 0.25% trypsin and had a molecular weight greater than 14 kDa. The cytotoxic component identified by these workers could not be neutralized using antisera raised against *E. coli*

verotoxins or *C. difficile* toxin (58). A subsequent report indicated the presence of a Shiga-like cytotoxin from *C. jejuni* which could be neutralized with monoclonal antibodies directed against the B subunit of the mature Shiga-toxin; however, these workers also detected a cytotoxin which could not be neutralized by the same monoclonal antibody (77). In addition, Guerrant and colleagues (58), unlike Yeen *et al.* (76), found cytotoxic activity in sonicated whole bacterial cell preparations. In the present study cytotoxic activity was detected both in culture and in sonicated filtrates of whole *C. jejuni* strain 2483 bacterial cells.

A cytotoxic complex comprising a porin and LPS was isolated and characterized. Previous studies showed a cytotoxic factor present in LPS-rich fractions from *C. jejuni* (60); however, it was not known what role the LPS played in toxicity. Misawa *et al.* (78) found that the expression of their cytotoxin was elevated when the *C. jejuni* was grown in Brucella broth. However, contrary to the findings of these workers, it was determined in the present work that HEp-2 cells showed the highest sensitivity to the cytotoxic complex and that these activities were consistently higher whether or not the cell cultures were grown in media supplemented with FBS (78). The increase in activities observed in the different cell lines may be due to the relative amounts of the receptor required for binding of the porin-LPS complex. Previous reports have implicated LPS in the adhesion of *C. jejuni* to epithelial cells as well as to intestinal mucus and also showed that this process could be inhibited by periodate oxidation (49). Since the cytotoxic activity of the porin-LPS was maintained following treatment with periodate in both HEp-2 and HeLa cells, it would appear that adhesion of the toxic complex is facilitated by components other than LPS. It is possible that expression of the porin protein may be

involved in binding the organism to host cells; however, Fauchere *et al.* (79) indicated that the MOMP was not involved in adherence to HeLa cells. From these studies it would appear that, although the LPS mediates attachment of organisms to host cells (49), the porin component binds the cytotoxic complex.

Although the mode of action of the cytotoxic porin remains unclear, the morphological changes induced by it are similar in nature to other well characterized bacterial cytotoxins. During early stages of intoxication the cytotoxic porin induced vacuole formation in HEp-2 and HeLa cells and this was similar in appearance to those produced in response to *H. pylori* vacuolating toxin (80). Even though vacuolation following intoxication with *C. jejuni* cytotoxin was shown here, no PCR products were generated using primers specific for *cagA* and *vacA* genes (72) suggesting that the genes encoding vacuole induction by the *C. jejuni* porin are unique from the genes carried and expressed in *H. pylori* .

When intoxication of host cells with the *C. jejuni* cytotoxic porin was extended beyond 24 h, vacuoles dissipated while the cytoplasmic blebbing and nuclear condensation typical of verotoxin and diphtheria toxin became more evident. Verotoxin and diphtheria toxin are both known to interfere with protein synthesis leading to programmed cell death or apoptosis (81,82). PCR-based screening of *C. jejuni* using verotoxin-specific primers was negative and confirmed the low stringency hybridization experiments of Moore *et al.* (77) and this suggested that the *C. jejuni* cytotoxic complex was distinct from verotoxin. It is possible that the porin from *C. jejuni* induces holes in the cell membrane in a manner similar to that resulting from *Staphylococcus aureus* α-toxin (82). Recently, the cytotoxic effects elicited by *Salmonella* Typhimurium porin suggested that

porins directly affect the cytoskeleton and the membrane ultrastructure of HEp-2 cells (83). In addition, porins from *Neisseria sp.* have also been shown to inhibit polymerization of actin in human neutrophils (84) while porins from *S. Typhimurium* have been found to induce both an inflammatory response (85) and the release of cytokines from human monocytes and lymphocytes (86).

Attempts to determine the isoelectric point of the cytotoxic protein by Coomassie blue staining and probing by Western blots of isoelectric focusing (IEF) gels with antisera raised against the complex were unsuccessful. Isolation of the cytotoxic porin protein using a chromatofocusing column and polybuffer 7-4 (Pharmacia Biotech) was difficult to reproduce due to probable interference from LPS. The isolation protocol was also applied to a cell-free filtrate from *C. jejuni* strain 3969 which had previously been reported to produce a cytotoxin (59,78,87). Although the strain produced a lower cytotoxic activity, similar morphological changes were observed in HEp-2 cells and a protein of similar size was observed following SDS-PAGE. A protein of comparable molecular weight was also present in crude concentrated filtrates from other cytotoxic strains of *Campylobacter sp.*, indicating that the release of the porin-LPS complex was not unique to *C. jejuni* strain 2483. Carbohydrates were also present in the cytotoxic product isolated from *C. jejuni* strain 3969. Although this strain was untypeable with available Lior antisera, it proved to be a biotype 1, Penner serotype O:50. The differential in cytotoxic activity between strain 3969 (low toxin activity) and 2483 (high toxin activity) could be a growth-rate dependent phenomenon since the release of the porin-LPS complex may occur most avidly during cell death or may be lost during active replication of the organism. Strains with a higher growth rate could therefore produce quantitatively more complex (88). Recently a vacuolating

cytotoxin similar to that produced by *H. pylori* was detected in the stools of children with diarrhea, even though no etiologic diarrheal agent was identified (89). Although many organisms have been attributed with the ability to induce vacuole formation in host cells, this process may be porin-mediated following release from dead or dying organisms (88).

Lectin studies showed that the carbohydrate portion of the LPS which co-purified with the porin possessed terminally-linked mannose as well as sialic acid terminally-linked to α(2-3) galactose and galactose-ß(1-4)-N-acetylglucosamine complexed together with hybrid N-glycan structures. Based on the thermostable somatic (O) antigen, the strain of *C. jejuni* used in this study was type O:11. The positive result with the lectin MAA suggests that the strain may be related to serotype O:19 (90). The LPS from the O:19 serostrain of *C. jejuni* has core structures that mimic those present on G_{M1} and G_{D1a} gangliosides and other strains of O:19 have been linked to post infectious neuropathies (91). The presence of terminally-linked mannose in the LPS of the O:11 serotype in this study may have significance. Treatment of the isolated complex with sodium meta-periodate, neuraminidase and N-glycosidase F had no effect on the toxicity elicited by the complex, suggesting that the LPS is not an integral component of the cytotoxic activity but that it may play a protective role. Indeed, it is possible that it may have interfered with the enzymatic degradation by trypsin and may offer an explanation for the disparity in trypsin inactivation data of previous reports. Under native conditions, the LPS likely forms complexes with the porin and protects it from discrete staining with Commassie blue. The cytotoxic protein is only revealed by Coomassie blue or silver staining after boiling in sample buffer containing SDS and ß-mercaptoethanol prior to SDS-PAGE. Since the porin from *C. jejuni* has been classified as part

of the trimeric porin family, it was not unexpected that it must be heat denatured in order to resolve the protein component of the cytotoxic complex (63).

When characterization of the cytotoxic porin is more complete and the encoding gene has been cloned and sequenced, a fuller understanding of the role of the porin in clinical campylobacteriosis will be forthcoming. Such evaluations may suggest potential roles for the porin-LPS complex as a diagnostic tool for the detection of either the organism or its cytotoxin or additionally as a recombinant vaccine for prevention and control of *Campylobacter* disease.

EXAMPLE 3

Screenings were conducted of 23 strains of *C. jejuni* and 9 strains of related organisms for phenotypic expression of a cytotoxin and presence of *porA* using primers specific for the porin gene sequenced from *C. jejuni* strain 2483. The results are shown in Table 8 below.

Table 8

Organism	LCDC number	Source	Lior Serotype	Biotype	Toxin positive	PCR positive
C. jejuni	3454	human	4	Ia	+	+
	3969	NA	untypable	I	+	+
	4951	human	7	I	+	+
	4966	human	7	I	+	+
	6847	human	1	Ia	+	+
	7099	chicken	61		+	+
	7288	water	9	II	+	+
	8916	human	94	IIa	+	+
	9214	human	2	Ia	+	+
	9541	water	82	II	+	+
	9543	water	82	II	+	+
	9555	human	23	I	+	+
	10403	human	36	Ia	+	-
	10673	human	82	II	+	+
	14040	human	82	II	+	+
	14906	human	82	I	+	+
	15151	human	82	I	+	+
	16323	beef	82	I	+	+
	16334	human	82	II	+	+
	16336	human	82	II	+	+
	16388 (2483)	human	82	II	+	+
	1	NA	4	I	+	+
	2074	NA	36	II	+	-
C. lari	729	NA	31	I	+	-
C. coli	348	NA	14	I	+	-
C. sputorum subsp. *fecalis*	5754	NA	NT	NT	+	-
C. fetus subsp. *fetus*	7055	NA	NT	NT	+	-
C. hyointestinalis	8494	human	NT	NT	+	-
C. jejuni subsp. *doylei*	9365	NA	NT	NT	+	-
A. butzleri	13220	human	7	IIIA	+	-
E. coli VT1+	3787 (19)	NA	NT	NT	+	-
E. coli VT2+	H19	human	NT	NT	+	-

NT not tested; NA information not available

The above results show that the porin of this invention is conserved in *C. jejuni* and *C. coli* both by phenotypic expression (toxin positive) and genotypic presence (ICR positive). This is a significant advantage over the Bleser gene which is not highly conserved.

EXAMPLE 4

PorA from *C. jejuni* strain 2483 according to this invention was compared against *H. influenzae* P2 and *C. jejuni* FlaA. This was done by obtaining hydrophobic profiles and beta sheet propensities as determined by the method of Novotny (31) using the PC/Gene

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(ii) TITLE OF INVENTION: A PORIN GENE FROM CAMPYLOBACTER JEJUNI, RELATED PRODUCTS AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 31

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/CA98/
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/041,200
(B) FILING DATE: 25-MAR-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1450 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Campylobacter jejuni
(B) STRAIN: 2483

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTTCGGATT TAAAATTTTT ACTATTTTAA GTGCTTCTTA AGAAAAAACT CCAAATTTAT 60

GTGCTACAAT TACAATGTTT TATTAATTTT TGACAAGGAG AATTCTCATG AAACTAGTTA 120

AACTTAGTTT AGTTGCAGCT CTTGCTGCAG GTGCTTTTTC AGCAGCTAAC GCTACTCCAC 180

TTGAAGAAGC TATCAAAGAT GTTGATGTAT CAGGTGTATT AAGATACAGA TACGATACAG 240

GTAATTTTGA TAAAAATTTC GTTAACAACT CAAATTTAAA CAACAACAAA CAAGATCACA 300

AATATAGAGC ACAAGTTAAC TTCAGTGCTG CTATAGCTGA TAACTTCAAA GCTTTCATTC 360

AGTTTGACTA CAACGCTGTT GATGGTGGCA CTGGTGTTGA TAACGTAACA AATGCCGAAA 420

AAGGACTTTT TGTTCGTCAA TTATACTTAA CTTATACAAA TGAAGATGTT GCTACAAGTG 480

TAATCGCTGG TAAACAACAA TTAAACCTTA TCTGGACGGA TAACGCTATT GATGGTTTAG 540

TAGGAACAGG TATCAAAGTA GTAAACAACA GCATCGATGG TTTAACTCTA GCTGCTTTTG 600

CTGTAGATAG CTTTATGGCG GAAGAGCAAG GTGCAGATTT ATTAGGACAA AGTACTATAT 660

CTACAACACA GAAAGCAGCT CCTTTTAAAG TGGATTCAGT AGGAAATCTT TATGGTGCTG 720

CTGCTGTAGG TTCTTATGAT CTTGCTGGCG GACAATTTAA TCCACAATTA TGGTTAGCTT 780

ACTGGGATCA AGTAGCATTC TTCTATGCTG TAGATGCAGC TTATAGTACA ACTATCTTTG 840

ATGGAATCAA CTGGACACTT GAAGGTGCTT ACTTAGGAAA TAGCCTTGAT AGCGAACTTG 900

ATGATAAAAC ACACGCTAAT GGCAATTTAT TTGCTTTAAA AGGTAGCATT GAAGTAAATG 960

GTTGGGATGC TAGCCTTGGT GGTTTATACT ACGGTGATAA AGAAAAAGCT TCTACAGTCG 1020

TAATCGAAGA TCAAGGTAAT CTTGGTTCTT TACTTGCAGG TGAGGAAATT TTCTATACTA 1080

CTGGTTCAAG ACTAAATGGT GATACTGGTA GAAATATCTT CGGTTATGTA ACTGGTGGAT 1140

ATACTTTCAA CGAAACAGTT CGCGTTGGTG CTGACTTCGT ATATGGTGGA ACAAAAACAG 1200

AAGATACTGC TCATGTAGGT GGTGGTAAAA AACTTGAAGC TGTTGCAAGA GTAGATTACA 1260

AATACTCTCC AAAACTTAAC TTCTCAGCAT TCTATTCTTA TGTGAACCTA GATCAAGGTG 1320

TAAACACTAA TGAAAGTGCT GATCATAGCA CTGTAAGACT TCAAGCTCTT TACAAATTCT 1380

AAGAAGCTTT CAAGTCTAAC TTCAAGGCGG AGTTTTGCTC CGCCTTTTTT TATGCCTGAT 1440

TTTTAAAACT 1450

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 424 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Campylobacter jejuni
(B) STRAIN: 2483

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Leu Val Lys Leu Ser Leu Val Ala Ala Leu Ala Ala Gly Ala
1               5                   10                  15

Phe Ser Ala Ala Asn Ala Thr Pro Leu Glu Glu Ala Ile Lys Asp Val
            20                  25                  30

Asp Val Ser Gly Val Leu Arg Tyr Arg Tyr Asp Thr Gly Asn Phe Asp
        35                  40                  45

Lys Asn Phe Val Asn Asn Ser Asn Leu Asn Asn Asn Lys Gln Asp His
    50                  55                  60

Lys Tyr Arg Ala Gln Val Asn Phe Ser Ala Ala Ile Ala Asp Asn Phe
65                  70                  75                  80

Lys Ala Phe Ile Gln Phe Asp Tyr Asn Ala Val Asp Gly Gly Thr Gly
                85                  90                  95

Val Asp Asn Val Thr Asn Ala Glu Lys Gly Leu Phe Val Arg Gln Leu
            100                 105                 110

Tyr Leu Thr Tyr Thr Asn Glu Asp Val Ala Thr Ser Val Ile Ala Gly
        115                 120                 125

Lys Gln Gln Leu Asn Leu Ile Trp Thr Asp Asn Ala Ile Asp Gly Leu
    130                 135                 140

Val Gly Thr Gly Ile Lys Val Val Asn Asn Ser Ile Asp Gly Leu Thr
145                 150                 155                 160

Leu Ala Ala Phe Ala Val Asp Ser Phe Met Ala Glu Glu Gln Gly Ala
                165                 170                 175
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Asp Leu Leu Gly Gln Ser Thr Ile Ser Thr Thr Gln Lys Ala Ala Pro
            180                 185                 190

Phe Lys Val Asp Ser Val Gly Asn Leu Tyr Gly Ala Ala Ala Val Gly
        195                 200                 205

Ser Tyr Asp Leu Ala Gly Gly Gln Phe Asn Pro Gln Leu Trp Leu Ala
    210                 215                 220

Tyr Trp Asp Gln Val Ala Phe Phe Tyr Ala Val Asp Ala Ala Tyr Ser
225                 230                 235                 240

Thr Thr Ile Phe Asp Gly Ile Asn Trp Thr Leu Glu Gly Ala Tyr Leu
                245                 250                 255

Gly Asn Ser Leu Asp Ser Glu Leu Asp Asp Lys Thr His Ala Asn Gly
            260                 265                 270

Asn Leu Phe Ala Leu Lys Gly Ser Ile Glu Val Asn Gly Trp Asp Ala
        275                 280                 285

Ser Leu Gly Gly Leu Tyr Tyr Gly Asp Lys Glu Lys Ala Ser Thr Val
    290                 295                 300

Val Ile Glu Asp Gln Gly Asn Leu Gly Ser Leu Leu Ala Gly Glu Glu
305                 310                 315                 320

Ile Phe Tyr Thr Thr Gly Ser Arg Leu Asn Gly Asp Thr Gly Arg Asn
                325                 330                 335

Ile Phe Gly Tyr Val Thr Gly Gly Tyr Thr Phe Asn Glu Thr Val Arg
            340                 345                 350

Val Gly Ala Asp Phe Val Tyr Gly Gly Thr Lys Thr Glu Asp Thr Ala
        355                 360                 365

His Val Gly Gly Gly Lys Lys Leu Glu Ala Val Ala Arg Val Asp Tyr
    370                 375                 380

Lys Tyr Ser Pro Lys Leu Asn Phe Ser Ala Phe Tyr Ser Tyr Val Asn
385                 390                 395                 400

Leu Asp Gln Gly Val Asn Thr Asn Glu Ser Ala Asp His Ser Thr Val
                405                 410                 415

Arg Leu Gln Ala Leu Tyr Lys Phe
```

420

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1275 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Campylobacter jejuni
(B) STRAIN: 2483

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGAAACTAG TTAAACTTAG TTTAGTTGCA GCTCTTGCTG CAGGTGCTTT TTCAGCAGCT 60

AACGCTACTC CACTTGAAGA AGCTATCAAA GATGTTGATG TATCAGGTGT ATTAAGATAC 120

AGATACGATA CAGGTAATTT TGATAAAAAT TTCGTTAACA ACTCAAATTT AAACAACAAC 180

AAACAAGATC ACAAATATAG AGCACAAGTT AACTTCAGTG CTCCTATAGC TGATAACTTC 240

AAAGCTTTCA TTCAGTTTGA CTACAACGCT GTTGATGGTG GCACTGGTGT TGATAACGTA 300

ACAAATGCCG AAAAAGGACT TTTTGTTCGT CAATTATACT TAACTTATAC AAATGAAGAT 360

GTTGCTACAA GTGTAATCGC TGGTAAACAA CAATTAAACC TTATCTGGAC GGATAACGCT 420

ATTGATGCTT TAGTAGGAAC AGGTATCAAA GTAGTAAACA ACAGCATCGA TGGTTTAACT 480

CTAGCTGCTT TTGCTGTAGA TAGCTTTATG GCGGAAGAGC AAGGTGCAGA TTTATTAGGA 540

CAAAGTACTA TATCTACAAC ACAGAAAGCA GCTCCTTTTA AAGTGGATTC AGTAGGAAAT 600

CTTTATGGTG CTGCTGCTGT AGGTTCTTAT GATCTTGCTG GCGGACAATT TAATCCACAA 660

TTATGGTTAG CTTACTGGGA TCAAGTAGCA TTCTTCTATG CTGTAGATGC AGCTTATAGT 720

ACAACTATCT TTGATGGAAT CAACTGGACA CTTGAAGGTG CTTACTTAGG AAATAGCCTT 780

GATAGCGAAC TTGATGATAA AACACACGCT AATGGCAATT TATTTGCTTT AAAAGGTAGC 840

ATTGAAGTAA ATGGTTGGGA TGCTAGCCTT GGTGGTTTAT ACTACGGTGA TAAAGAAAAA 900

GCTTCTACAG TCGTAATCGA AGATCAAGGT AATCTTGGTT CTTTACTTGC AGGTGAGGAA 960

ATTTTCTATA CTACTGGTTC AAGACTAAAT GGTGATACTG GTAGAAATAT CTTCGGTTAT 1020

GTAACTGGTG GATATACTTT CAACGAAACA GTTCGCGTTG GTGCTGACTT CGTATATGGT 1080

GGAACAAAAA CAGAAGATAC TGCTCATGTA GGTGGTGGTA AAAAACTTGA AGCTGTTGCA 1140

AGAGTAGATT ACAAATACTC TCCAAAACTT AACTTCTCAG CATTCTATTC TTATGTGAAC 1200

CTAGATCAAG GTGTAAACAC TAATGAAAGT GCTGATCATA GCACTGTAAG ACTTCAAGCT 1260

CTTTACAAAT TCTAA 1275

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTCTCCCTTC TCGAATCGTA ACCGTTCGTA CGAGAATCGC TGTCCTCTCC TTC 53

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATCGAAGGA GAGGACGCTG TCTGTCGAAG GTAAGGAACG GAGGAGAGAA GGGAGAG 57

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATTGAAGGA GAGGACGCTG TCTGTCGAAG GTAAGGAACG GAGGAGAGAA GGGAGAG 57

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGCTGAAGGA GAGGACGCTG TCTGTCGAAG GTAAGGAACG GAGGAGAGAA GGGAGAG 57

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTAGGAAGGA GAGGACGCTG TCTGTCGAAG GTAAGGAACG GAGGAGAGAA GGGAGAG 57

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGAATCGTAA CCGTTCGTAC GAGAATCGCT 30

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTAATTTTG ATAAAAATTT 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATACAGGTA AATTTGATAA 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GAAGAAGCTA TCAAAGATGT 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGCCACCATC AACAGCGTTG 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TAAGTAAGCA CCTTCAAGTG 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ACTTGTGCTC TATATTTGTG 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGATAGCGAA CTTGATGATA 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AGCATCCCAA CCATTTACTT 20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TGACTTCGTA TATGGTGGAA 20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CTCCAAATTT ATGTGCTACA 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTATCAAATT TCCAACTTCT 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TGAAGATGTT GCTACAAGTG 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTACTCTTGC AACAGCTTCA 20

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CTTCAAAGCT TTCATTCAGT 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GAAGAGTCCG TGGGATTACG 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGCGATGCAG CTATTAATAA 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

TTAACCACAC CCACCGCAGT 20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GCTCTGGATG CATCTCTGGT 20

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

AGTAAGGAGA AACAATGA 18

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

AATAAGCCTT AGAGTCTTTT TGGAATCC 28

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GCTTCTCTTA CCACCAATGC 20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

TGTCAGGGTT GTTCACCATG 20

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CLAIMS:

1. An isolated and purified nucleic acid, characterized in that said nucleic acid encodes a *porA* protein of *Campylobacter jejuni*, or an antigenic fragment thereof.

2. A nucleic acid according to claim 1, characterized in that said nucleic acid encodes a 424 amino acid cytotoxic protein having a calculated molecular weight of 45.6 kDa and a pI of 4.44.

3. A nucleic acid according to claim 1, characterized in that it is derived from strain 2483 of *Campylobacter jejuni* (ATCC Accession No.).

4. A nucleic acid according to claim 1, characterized in that it encodes a protein having an amino acid sequence SEQ ID NO:2, wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the cytotoxic characteristic of the protein.

5. A nucleic acid according to claim 1, characterized in that said nucleic acid is of SEQ ID NO:3, wherein said nucleotide sequence encompasses nucleotide substitutions, additions and deletions that do not alter the cytotoxic characteristic of the encoded protein.

6. A purified cytotoxic protein encoded by at least a portion of said nucleic acid of claim 1, claim 2, claim 3, claim 4 or claim 5.

7. A purified protein according to claim 6, characterized by amino acid sequence SEQ ID NO:2.

8. A DNA probe, characterized in that said probe has a nucleotide sequence corresponding to a part of a target

sequence SEQ ID NO:1, wherein the nucleotide sequence of the probe encompasses nucleotide substitutions, additions and deletions that do not affect the ability of the probe to bind specifically to said target.

9. A method of detecting the presence of Campylobacter jejuni infection, characterized by the steps of:

a) contacting a sample obtained from a patient suspected of infection, with a detectable amount of a protein of claim 6 or claim 7, for a time sufficient to allow formation of a complex between said protein and any anti-*Campylobacter jejuni* antibodies present in said sample; and

b) detecting the presence of, and optionally the quantity of, said complex formed during step (a).

10. A method of detecting the presence of *Campylobacter jejuni* in a patient, characterized by obtaining from said patient a sample suspected of containing *Campylobacter jejuni*, and detecting whether the characteristic nucleic acid of claim 1, claim 2, claim 3, claim 4 or claim 5 is contained in said sample.

11. The method of claim 10, wherein the nucleic acid is detected by amplifying any of said characteristic nucleic acid present in said sample, and then detecting the amplified nucleic acid.

12. The method of claim 11, wherein the amplification is achieved by polymerase chain reaction.

13. A pharmaceutical composition comprising a pharmaceutically acceptable diluent or carrier and the antigenic protein of claim 6 or claim 7 or an antigenic fragment thereof.

14. An isolated expression vector, characterized by a region encoding a *porA* protein of *Campylobacter jejuni*, or an antigenic fragment thereof.

15. A vector according to claim 14, characterized in that said region encodes SEQ ID NO:3.

16. A host transformed or transfected with the expression vector of claim 14 or claim 15.

17. A kit for practicing the method of claim 9, comprising a receptacle for said sample, a container holding said polypeptide, and a means for detecting said complex.

18. A kit for practicing the method of claim 10 comprising a receptacle for a container holding said antibodies, and a means for detecting said complex.

19. A vaccine comprising an immunogenically effective amount of the *porA* antigen of *Campylobacter jejuni* or antigenic fragment thereof and a pharmaceutically acceptable carrier.

20. A vaccine, characterized in that it contains a protein having amino acid sequence SEQ ID NO:2, wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the ability of the protein to raise antibodies when introduced into a human or animal body.

21. A method of inducing an immune response in a human or animal host by administering to the host a foreign protein, characterized in that said protein has an amino acid sequence SEQ ID NO:2, wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the ability of the protein to

raise antibodies when introduced into said human or animal body.

22. A method of producing antibodies for testing for infection by *Campylobacter jejuni*, characterized in that a protein having an amino acid sequence of SEQ ID NO:2 is introduced into a human or animal body to raise antibodies, and said antibodies are subsequently isolated from said body, wherein said amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the ability of the protein to raise antibodies when introduced into said human or animal body.

Applicant's or agent's file reference number 4..07-PT	International application PCT/CA98/00272

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 8, lines 20 and 21.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive,
Rockville, MD 20852
U.S.A.

Date of deposit	Accession Number
March 19, 1998	ATCC 202,101

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

Microorganisms to be made available to public only by issuance of sample to an expert nominated by Applicant prior to issuance or abandonment.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

EPO
Canada
Other countries permitting such restriction

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[ ] This sheet was received with the international application	[✓] This sheet was received by the International Bureau on: 23 JUNE 1998
Authorized officer Maureen Matheson	Authorized officer Paul Rez

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Applicant's or agent's file reference number ,1207-PT	International application PCT/CA98/00272

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 49, lines 6 to 10

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive,
Rockville, MD 20852
U.S.A.

Date of deposit	Accession Number
March 19, 1998	ATCC 202,102

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

Microorganisms to be made available to public only by issuance of sample to an expert nominated by Applicant prior to issuance or abandonment.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

EPO
Canada
Other countries permitting such restriction

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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Authorized officer Maureen Matheson	Authorized officer

Form PCT/RO/134 (July 1992)

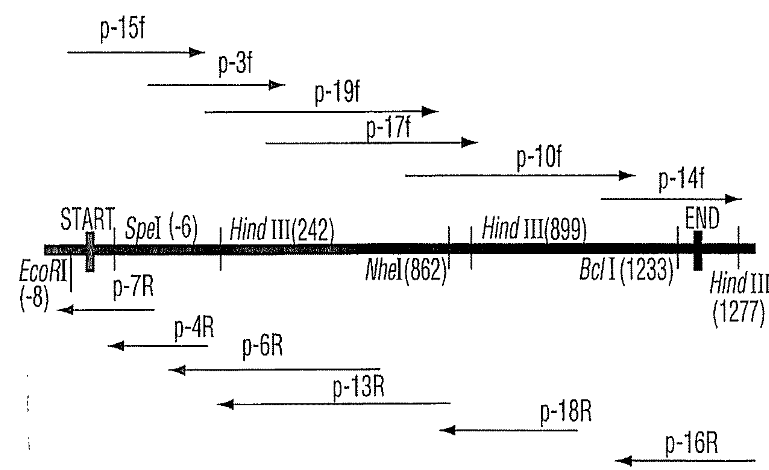

FIG. 1

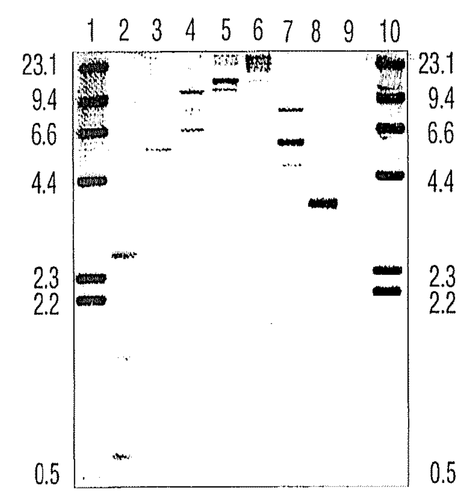

FIG. 2

```
     CCTTCGGATTTAAAATTTTTACTATTTTAAGTGCTTCTTAAGAAAAAACTCCAAATTTAT   60
                                   -35                   -10

61   GTGCTACAATTACAATGTTTTATTAATTTTTGACAAGGAGAATTCTCATGAAACTAGTTA  120
                                          RBS        M  K  L  V  K    5

121  AACTTAGTTTAGTTGCAGCTCTTGCTGCAGGTGCTTTTTCAGCAGCTAACGCTACTCCAC  180
      L  S  L  V  A  A  L  A  A  G  A  F  S  A  A  N  A  T  P  L    25

181  TTGAAGAAGCTATCAAAGATGTTGATGTATCAGGTGTATTAAGATACAGATACGATACAG  240
      E  E  A  I  K  D  V  D  V  S  G  V  L  R  Y  R  Y  D  T  G    45

241  GTAATTTTGATAAAAATTTCGTTAACAACTCAAATTTAAACAACAACAAACAAGATCACA  300
      N  F  D  K  N  F  V  N  N  S  N  L  N  N  N  K  Q  D  H  K    65

301  AATATAGAGCACAAGTTAACTTCAGTGCTGCTATAGCTGATAACTTCAAAGCTTTCATTC  360
      Y  R  A  Q  V  N  F  S  A  A  I  A  D  N  F  K  A  F  I  Q    85

361  AGTTTGACTACAACGCTGTTGATGGTGGCACTGGTGTTGATAACGTAACAAATGCCGAAA  420
      F  D  Y  N  A  V  D  G  G  T  G  V  D  N  V  T  N  A  E  K   105

421  AAGGACTTTTTGTTCGTCAATTATACTTAACTTATACAAATGAAGATGTTGCTACAAGTG  480
      G  L  F  V  R  Q  L  Y  L  T  Y  T  N  E  D  V  A  T  S  V   125

481  TAATCGCTGGTAAACAACAATTAAACCTTATCTGGACGGATAACGCTATTGATGGTTTAG  540
      I  A  G  K  Q  Q  L  N  L  I  W  T  D  N  A  I  D  G  L  V   145

541  TAGGAACAGGTATCAAAGTAGTAAACAACAGCATCGATGGTTTAACTCTAGCTGCTTTTG  600
      G  T  G  I  K  V  V  N  N  S  I  D  G  L  T  L  A  A  F  A   165

601  CTGTAGATAGCTTTATGGCGGAAGAGCAAGGTGCAGATTTATTAGGACAAAGTACTATAT  660
      V  D  S  F  M  A  E  E  Q  G  A  D  L  L  G  Q  S  T  I  S   185

661  CTACAACACAGAAAGCAGCTCCTTTTAAAGTGGATTCAGTAGGAAATCTTTATGGTGCTG  720
      T  T  Q  K  A  A  P  F  K  V  D  S  V  G  N  L  Y  G  A  A   205

721  CTGCTGTAGGTTCTTATGATCTTGCTGGCGGACAATTTAATCCACAATTATGGTTAGCTT  780
      A  V  G  S  Y  D  L  A  G  G  Q  F  N  P  Q  L  W  L  A  Y   225

781  ACTGGGATCAAGTAGCATTCTTCTATGCTGTAGATGCAGCTTATAGTACAACTATCTTTG  840
      W  D  Q  V  A  F  F  Y  A  V  D  A  A  Y  S  T  T  I  F  D   245

841  ATGGAATCAACTGGACACTTGAAGGTGCTTACTTAGGAAATAGCCTTGATAGCGAACTTG  900
      G  I  N  W  T  L  E  G  A  Y  L  G  N  S  L  D  S  E  L  D   265

901  ATGATAAAACACACGCTAATGGCAATTTATTTGCTTTAAAAGGTAGCATTGAAGTAAATG  960
      D  K  T  H  A  N  G  N  L  F  A  L  K  G  S  I  E  V  N  G   285

961  GTTGGGATGCTAGCCTTGGTGGTTTATACTACGGTGATAAAGAAAAAGCTTCTACAGTCG 1020
      W  D  A  S  L  G  G  L  Y  Y  G  D  K  E  K  A  S  T  V  V   305

1021 TAATCGAAGATCAAGGTAATCTTGGTTCTTTACTTGCAGGTGAGGAAATTTTCTATACTA 1080
      I  E  D  Q  G  N  L  G  S  L  L  A  G  E  E  I  F  Y  T  T   325
```

FIG. 3


```
1081 CTGGTTCAAGACTAAATGGTGATACTGGTAGAAATATCTTCGGTTATGTAACTGGTGGAT 1140
      G  S  R  L  N  G  D  T  G  R  N  I  F  G  Y  V  T  G  G  Y   345

1141 ATACTTTCAACGAAACAGTTCGCGTTGGTGCTGACTTCGTATATGGTGGAACAAAAACAG 1200
      T  F  N  E  T  V  R  V  G  A  D  F  V  Y  G  G  T  K  T  E   365

1201 AAGATACTGCTCATGTAGGTGGTGGTAAAAAACTTGAAGCTGTTGCAAGAGTAGATTACA 1260
      D  T  A  H  V  G  G  G  K  K  L  E  A  V  A  R  V  D  Y  K   385

1261 AATACTCTCCAAAACTTAACTTCTCAGCATTCTATTCTTATGTGAACCTAGATCAAGGTG 1320
      Y  S  P  K  L  N  F  S  A  F  Y  S  Y  V  N  L  D  Q  G  V   405

1321 TAAACACTAATGAAAGTGCTGATCATAGCACTGTAAGACTTCAAGCTCTTTACAAATTCT 1380
      N  T  N  E  S  A  D  H  S  T  V  R  L  Q  A  L  Y  K  F  *   424

1381 AAGAAGCTTTCAAGTCTAACTTCAAGGCGGAGTTTTGCTCCGCCTTTTTTTATGCCTGAT 1440

               1441   TTTTAAAACT   1450
```

Fig. 3 (cont'd)

```
                                   
C. jejuni PorA        MKlvkLsLva alaagafsAa nAtpleeaik dvDVsGvlry rydtgnfDkn
H. influenzae P2                 .mKkTLAalI vGafAasaAn AAvVYNnEGt nvElgGrlsi iaeqSnstvd
E. cloacae PhoE                  MKKSTLALvV MGvVAsAsvh AAEVYNKnGN KLDVYGKVKA MHYiSDdDtK
K. pneumoniae PhoE    MKKSTLALmm MGfVAstatq AAEVYNKnaN KLDVYGKIKA MHYFSDyDSK
S. typhi OmpC         MKvkvLsLlV pallvagaAn AAEIYNKDGN KLDlFGKVdg LHYFSDdkgs
E. coli PhoE          MKKSTLALvV MGiVAsAsvq AAEIYNKDGN KLDVYGKVKA MHYmSDnaSK

                                 51
C. jejuni PorA        fvnnsnlnnn kQdhkYraqv nfsaaIaDnF kaFiqFDyna vdggTgvDnv
H. influenzae P2                 nqkQqhgalr nQgsRF..hi KathnfgDgF yaqGyLEtrF vtkasEngSd
E. cloacae PhoE                  DGDQTYV... ....RF..Gf KGETQINDQL TGYGRWEaEF aGNKaESDS.
K. pneumoniae PhoE    DGDQTYV... ....RF..Gl KGETQINEdL TGYGRWEsEF sGNKTESDS.
S. typhi OmpC         DGDQTYm... ....Ri..Gf KGETQVNDQL TGYGqWEyqi qGNqTE.gS.
E. coli PhoE          DGDQsYI... ....RF..Gf KGETQINDQL TGYGRWEaEF aGNKaESDt.

                                 101
C. jejuni PorA        tnaEkglfvr qlyltyTned vAtsviagkq qLnliWtdnA idglvgtgik
H. influenza P2                  nfgDit.... ......skyA YvtLgnKaFG evklGRakti adgItsaeD.
E. cloacae PhoE                  ..s.QK.... ......TRLA FAGLKLKDFG SLDYGRNLGA LYDVEAWTDM
K. pneumoniae PhoE    ..sqQK.... ......TRLA FAGvKLKnYG SFDYGRNLGA LYDVEAWTDM
S. typhi OmpC         ..nDsw.... ......TRvA FAGLKFaDaG SFDYGRNyGv tYDVtsWTDv
E. coli PhoE          ..aqQK.... ......TRLA FAGLKYKDLG SFDYGRNLGA LYDVEAWTDM

                                 151
C. jejuni PorA        vvnnsidglt LaaFavDSfm aeeQgadLLg qstisttqkA apFkvdsvgn
H. influenzae P2                 .......... .kEYGv.... ..lnnsdYip tsgntv...g yTFk...giD
E. cloacae PhoE                  .......... FPEFGGDSS. ..AQTDNFMT KRASGL...A .TYR...NTD
K. pneumoniae PhoE    .......... FPEFGGDSS. ..AQTDNFMT KRASGL...A .TYR...NTD
S. typhi OmpC         .......... LPEFGGDty. ..g.aDNFMq qRgnGy...A .TYR...NTD
E. coli PhoE          .......... FPEFGGDSS. ..AQTDNFMT KRASGL...A .TYR...NTD

                                 201
C. jejuni PorA        LYGaaavgsy dLaggqfNpq lwlaywDqva ffyavdaaYs Tti...FdGi
H. influenzee P2                 gLvLganylL aqkreGakGe nkrpNdkagE vrigeinn.G iqvgakYdan
E. cloacae PhoE                  FFGaIDGLDM TLQYQGKN.. ......EnRD aKKQNGDGFG TSLTYDFGGT
K. pneumoniae PhoE    FFGLVDGLDL TLQYQGKN.. ......EgRE aKKQNGDGvG TSLsYDFGGT
S. typhi OmpC         FFGLVDGLDF aLQYQGKNGs vsgeNtngRs llnQNGDGYG gSLTYaiGe.
E. coli PhoE                     FFGvIDGLnL TLQYQGKN.. ......EnRD vKKQNGDGFG TSLTYDFGGs

                                 251
C. jejuni PorA        nWtleGAYlg nsldseldDk ThangnLfal kgsievngwD AslgGLyYgd
H. influenza P2                  DivakiAY.. ........gR TNykynesde hkqqlngvla tlgyrFsdlg
E. cloacae PhoE                  DFAVSGAYTN S.......DR TNaQNlLARA ....QGqkAE AWATGLKYDA
K. pneumoniae PhoE    DFAVSaAYTs S.......DR TNDQNlLARA ....QGskAE AWATGLKYDA
S. typhi OmpC         gFsVgGAiTt S.......kR TaDQNntAnA rlygnGdrAt vYtgGLKYDA
E. coli PhoE          DFAISGAYTN S.......DR TNEQNlqsRg ....tGkrAE AWATGLKYDA

                                 301
C. jejuni PorA        kekastvvie DqgNLgslla geeifytTgs rLngdtgrni fgyVtggYtF
H. influenzae P2                 llVsLdsgYa kTkNyk.... .......... ..ikhekrYf ...VspgFQY
E. cloacae PhoE                  NdIYLAaMYS ETRNMTPISG .......... GFANKAQNFE ...VVAQYQF
K. pneumoniae PhoE    NNIYLAtMYS ETRkMTPISG .......... GFANKAQNFE ...aVAQYQF
S. typhi OmpC         NNIYLAaqYS qTyNaTrfgt sngsnpsTsy GFANKAQNFE ...VVAQYQF
E. coli PhoE          NNIYLAtfYS ETRkMTPItG .......... GFANKtQNFE ...aVAQYQF
```

FIG. 4


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                                 351
C. jejuni PorA        netvRvgadF VyggtKtedt ahvg..Ggkk LeavarVdyk YspklNFSAF
H. influenzae P2                 EL.MedtnvY gnfKyertSv dqGEktrEqa vl....fGvd hkLhKqLltY
E. cloacae PhoE                  DFGLRPSLGY VqSKGKDn.. ...EGIGDED LVkYIDVGAT YYFNKNMSAF
K. pneumoniae PhoE    DFGLRPSLGY VlSKGKDI.. ...EGVGsED LVnYIDVGlT YYFNKNMnAF
S. typhi OmpC         DFGLRPSvaY lqSKGKDISn gyGasyGDqD iVkYVDVGAT YYFNKNMStY
E. coli PhoE          DFGLRPSLGY VlSKGKDI.. ...EGIGDED LVnYIDVGAT YYFNKNMSAF

                                 401                                         434
C. jejuni PorA        ysY....vnl Dqgvntnesa DhstVrlqal YkF*
H. influenzae P2                 IEgayartrt tetgkgvkte kEksVgvGLr vyF*
E. cloacae PhoE                  VDYKINQiDd DN...KLGVs sDDIVAvGMT YQF*
K. pneumoniae PhoE    VDYKINQLkS DN...KLGIN DDDIVAlGMT YQF*
S. typhi OmpC         VDYKINlLDk ndftrdaGIN tDDIVAlGLv YQF*
E. coli PhoE          VDYKINQLDS DN...KLnIN nDDIVAvGMT YQF*
```

FIG. 4 (cont'd)

FIG. 5

```
              T
             T T
             T G
             G:C
             A:T
    1410 -   G:C  - 1420
             G:C
             C:G
             G:C
             G:C
    1405 -   A:T  - 1425
             A:T
             / \
GTCTAACTTC      TTTTTATGCC
```

FIG. 6A

FIG. 6B

SUBSTITUTE SHEET (RULE 26)

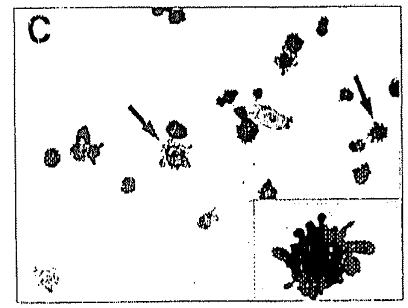

FIG. 6C

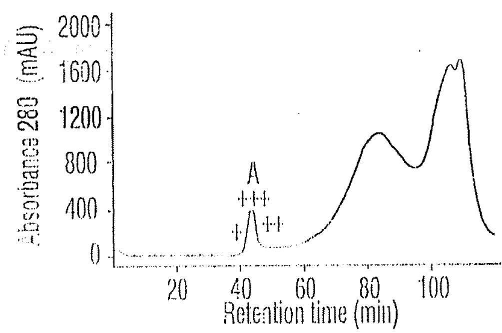

FIG. 7A

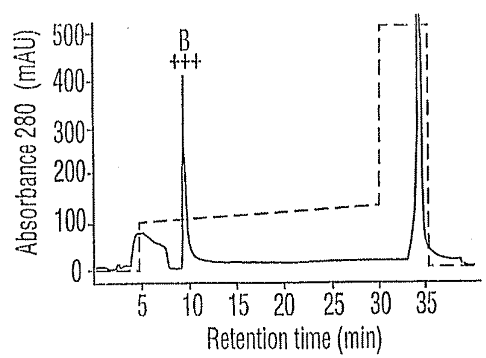

FIG. 7B

SUBSTITUTE SHEET (RULE 26)

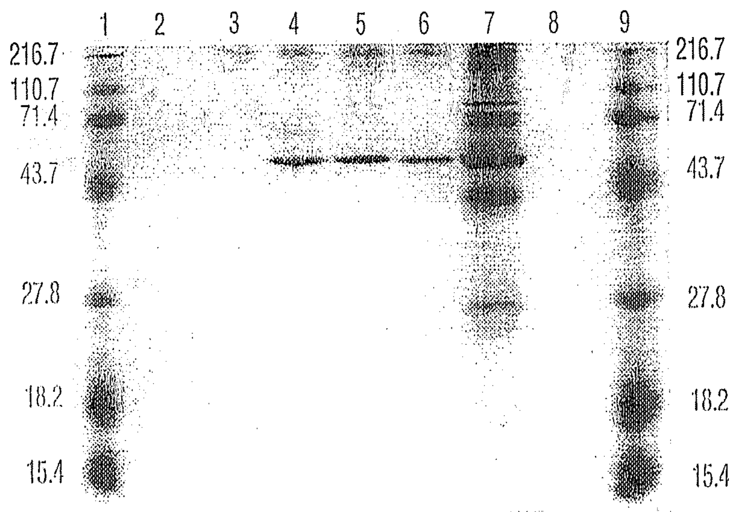

FIG. 8

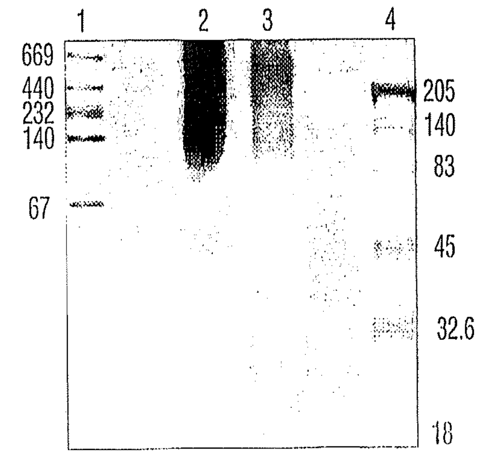

FIG. 9

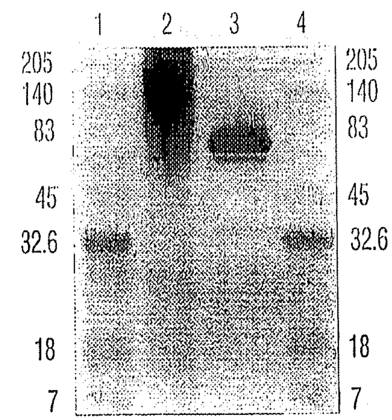

FIG. 10

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Por A from C. *Jejuni* strain 2483

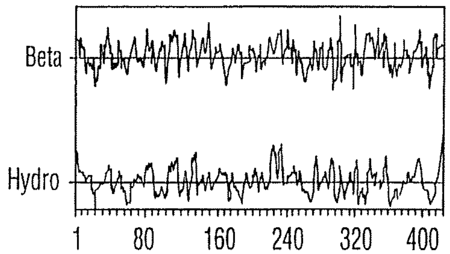

FIG. 11A

H. influenzea P2

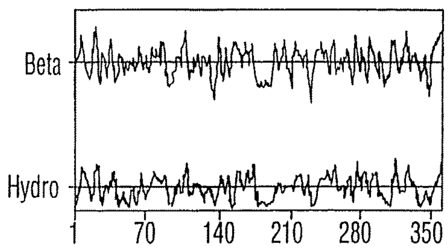

FIG. 11B

C. Jejuni FlaA

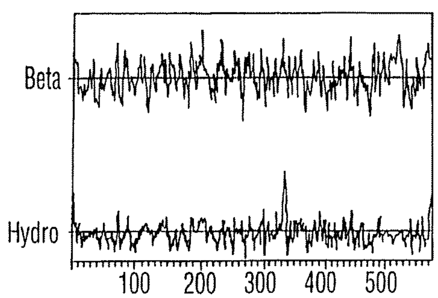

FIG. 11C

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 98/00272

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/205 C12Q1/68 G01N33/68 A61K39/106 C07K16/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOLLA J-M ET AL: "Conformational analysis of the Campylobacter jejuni porin." JOURNAL OF BACTERIOLOGY 177 (15). 1995. 4266-4271. ISSN: 0021-9193, XP002071484 cited in the application see the whole document ---	1-22
X	ZHUANG J ET AL: "The Campylobacter jejuni porin trimers pack into different lattice types when reconstituted in the presence of lipid." EUROPEAN JOURNAL OF BIOCHEMISTRY 244 (2). 1997. 575-579. ISSN: 0014-2956, XP002071483 see the whole document ---	1-22
	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
15 July 1998	28.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HUYER M ET AL: "OUTER MEMBRANE PORIN PROTEIN OF CAMPYLOBACTER -JEJUNI." FEMS (FED EUR MICROBIOL SOC) MICROBIOL LETT 37 (3). 1986. 247-250. CODEN: FMLED7 ISSN: 0378-1097, XP002071485 cited in the application see the whole document ---	
A	PAGE W J ET AL: "CHARACTERIZATION OF THE PORINS OF CAMPYLOBACTER -JEJUNI AND CAMPYLOBACTER -COLI AND IMPLICATIONS FOR ANTIBIOTIC SUSCEPTIBILITY." ANTIMICROB AGENTS CHEMOTHER 33 (3). 1989. 297-303. CODEN: AMACCQ ISSN: 0066-4804, XP002071486 see the whole document ---	
A	CHART H ET AL: "Outer membrane characteristics of Campylobacter jejuni grown in chickens." FEMS MICROBIOLOGY LETTERS 145 (3). 1996. 469-472. ISSN: 0378-1097, XP002071487 see the whole document ---	
A	AMAKO K ET AL: "Electron microscopy of the major outer membrane protein of Campylobacter jejuni." MICROBIOLOGY AND IMMUNOLOGY 40 (10). 1996. 749-754. ISSN: 0385-5600, XP002071488 see the whole document ---	
P,X	BACON, DAVID JOHN: "Molecular characterization of a cytotoxic porin protein from Campylobacter jejuni and its role in campylobacteriosis (enteritis, virulence)" (1997) 171 PP. AVAIL.: UMI, ORDER NO. DA9730821 FROM: DISS. ABSTR. INT., B 1997, 58(4), 1665, October 1997, XP002071489 see the whole document ---	1-22
X,P	W. SCHRÖDER ET AL.: "Primary structure analysis and adhesion studies on the major outer membrane protein of Campylobacter jejuni." FEMS MICROBIOLOGY LETTERS, vol. 150, 1997, pages 141-147, XP002071482 see the whole document ---	1-22

-/--

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	MOSER I ET AL: "Campylobacter jejuni major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes." FEMS MICROBIOLOGY LETTERS, vol. 157, 1997, pages 233-238, XP002071491 see the whole document -----	1-22

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 98/00272

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 21 and 22 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.